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## SOME DERIVATIVES OF CHOLESTEROL.

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(Received for publication, July 3, 1928.)

### INTRODUCTION.

Because of its antihemolytic effect (1) attempts have been made to incorporate cholesterol (2) in certain serums and vaccines in order to prevent any hemolysis of red blood corpuscles when such solutions are injected. Owing to the insolubility of cholesterol, however, difficulty has been experienced in preparing solutions containing cholesterol. The object of the present work was to prepare a water-soluble derivative of cholesterol.

Cholesterol is a complex unsaturated secondary alcohol (3). In order to retain the antihemolytic effect the derivative chosen should not change the cholesterol molecule and should be hydrolyzable to cholesterol. The particular derivative sought was the *p*-aminobenzoate of cholesterol. It was hoped that salts of this amine would be water-soluble.

This derivative would also be of interest because of the possibility of local anesthetic action. Several of the simple esters of *p*-aminobenzoic acid (4) possess local anesthetic action, and the union of this grouping with cholesterol, a substance occurring in nervous tissue, might produce a compound possessing marked anesthetic action.

The same derivatives of dihydrocholesterol were also prepared in order to compare their properties with the corresponding compounds of the original unsaturated sterol.

Anderson (5) has found that cholesterol from different sources possesses slight differences in physical properties. The compounds prepared in the present work might also be of value in fractionating cholesterol itself and would be of service in identification and comparison of cholesterol with other reduced and unreduced sterols isolated from plants (6). It was for this reason



## Some Derivatives of Cholesterol

that the preparation of the previously described benzoates was repeated, and the optical rotations determined, since the fractionation of sterols can be followed most easily by noting the change in specific rotation.

### DISCUSSION.

The method chosen for the preparation of these derivatives consisted in condensing the sterol with *p*-nitrobenzoyl chloride followed by reduction of the resulting *p*-nitrobenzoate. Considerable difficulty was experienced in this reduction. Owing to its insolubility it could not be reduced by any of the usual reducing agents, iron, tin, and amalgamated zinc with hydrochloric acid all having no effect.

TABLE I.  
*Properties of Derivatives.*

Substance .....	Cholesterol.		Dihydrocholesterol.	
	M.p. (corrected).	$[\alpha]_D^{25}$ CHCl <sub>3</sub> .	M.p. (corrected).	$[\alpha]_D^{25}$ CHCl <sub>3</sub> .
	°C.	degrees	°C.	degrees
Free sterol.....	149-150	-39.25	142-143	+26.35
Benzoate.....	144-145	-13.70	135.8-136.8	+21.40
<i>p</i> -Nitrobenzoate.....	188.8-189.8	-6.48	156.5-157.7	+20.05
<i>p</i> -Aminobenzoate.....	237.4-238.5	+3.61	191-192	+26.55

It was found that the *p*-nitrobenzoate could be easily reduced to the *p*-aminobenzoate by selective catalytic reduction. A solution of the nitro compound in ethyl acetate was shaken with hydrogen under pressure in the presence of the platinum oxide-platinum black (7) catalyst. By allowing the reduction to run for a short time, only the nitro group was reduced and the double bond in the sterol remained untouched.

The properties of the various derivatives prepared may be summarized in Table I.

From Table I it will be noted that the rotations of the cholesterol derivatives increase from the  $-39.25^\circ$  of the free sterol to  $+3.61^\circ$  of the *p*-aminobenzoate. The latter fact is most remarkable since from a consideration of the relative molecular weights the rotation of the amino compound should fall between that of the

benzoate and the *p*-nitrobenzoate. It was proved that this dextrorotation was not due to a reduction of the sterol molecule since saponification yielded free cholesterol with the same rotation as the original. A comparison of the rotations and melting points of the *p*-aminobenzoates of cholesterol and dihydrocholesterol also indicates that no reduction of the double bond had taken place.

The nitro group is reduced completely in 25 minutes, whereas about 4 hours are required to reduce cholesterol to dihydrocholesterol. The latter reduction is quite difficult and requires a larger amount of catalyst. In previous work on the reduction of sterols (8), ether, alcohol, or glacial acetic acid was used as the solvent. In the present work a careful study of different solvents led to the use of ethyl acetate. This solvent permits the reduction of 4 to 5 gm. of sterol in 4 hours, whereas glacial acetic acid requires 4 to 5 days for 1 gm. Moreover, the reduced sterol obtained from the reduction with ethyl acetate is *absolutely* negative to the Liebermann-Burchard reaction and possesses a rotation of  $+26.35^\circ$  which is considerably higher than that usually given. The rotations given in the literature range from  $+23^\circ$  to  $+34^\circ$ . The present value agrees with that obtained by Doree (9), and since the product gives no Liebermann-Burchard reaction whatever, it is believed to represent the rotation of the completely reduced sterol. The higher value,  $+34.00^\circ$  of Boehm (10), is undoubtedly incorrect.

All the above aminobenzoates were insoluble in water and dilute hydrochloric acid. The hydrochlorides were prepared by passing dry hydrogen chloride into ether solutions of the bases. These salts were very insoluble so no hemolytic indices could be determined. Neither the amines nor their hydrochlorides exhibited any local anesthetic action at all, due undoubtedly to their insolubility.

#### EXPERIMENTAL.

The cholesterol used for the preparation of the following derivatives was purified by the method of Anderson (5). After two crystallizations from alcohol colorless plates were obtained. M. p.  $149-50^\circ$ ,  $[\alpha]_D^{25} = -39.25^\circ$  in  $\text{CHCl}_3$ .

*Preparation of Cholesteryl Benzoate.*

5 gm. of dried cholesterol were heated with 2.5 gm. of benzoyl chloride at a temperature of  $160^{\circ}$  for 15 minutes. After cooling, the mass was disintegrated with water and then triturated with a large volume of alcohol which removes any unchanged cholesterol or benzoyl chloride. The insoluble portion, after filtration, was dissolved in ether and extracted twice with sodium bicarbonate solution and then with water. The ether solution was filtered, one-third its volume of alcohol was added, and the solution allowed to evaporate at room temperature. 4.5 gm. of colorless needles were obtained which melted at  $144-145^{\circ}$  after drying at  $105^{\circ}$  *in vacuo* over phosphorous pentoxide.  $[\alpha]_D^{25} = -13.70^{\circ}$  in  $\text{CHCl}_3$ .

Analysis. 0.1036 gm. substance: 0.3153 gm.  $\text{CO}_2$ , 0.0939 gm.  $\text{H}_2\text{O}$ .

$\text{C}_{32}\text{H}_{50}\text{O}_2$ . Calculated. C 83.26 per cent, H 10.20 per cent.

Found. " 82.81 " " " 10.14 " "

The benzoate has been previously prepared by Doree (9), M. p.  $150-151^{\circ}$ , and by Obermüller (11), m. p.  $145^{\circ}$ . No rotations were given. Recrystallization of the above product did not change the melting point or rotation.

*Preparation of Cholesteryl p-Nitrobenzoate.*

A solution of 5 gm. of dried cholesterol in 15 cc. of toluene and 3.5 gm. of *p*-nitrobenzoyl chloride were heated in an oil bath at  $165^{\circ}$  for 15 minutes. The toluene was distilled off and 20 cc. of alcohol added to remove any unchanged *p*-nitrobenzoyl chloride. After the mixture was filtered, the precipitate was dissolved in benzene and washed with dilute sodium bicarbonate solution to remove any *p*-nitrobenzoic acid. After the solution was filtered, alcohol was added until a precipitate first appeared and the solution allowed to evaporate at room temperature until light yellow needles of the ester crystallized out. When dried it melted at  $188.8-189.8^{\circ}$  and possessed a rotation in  $\text{CHCl}_3$  of  $-6.48^{\circ}$ .

Analysis (Kjeldahl). 0.1543 gm. substance required 27.14 cc. 0.01 N HCl.

$\text{C}_{32}\text{H}_{48}\text{O}_4\text{N}$ . Calculated. N 2.61 per cent. Found. N 2.46 per cent.

Doree and Orange (12) reported the *p*-nitrobenzoate as having a melting point of 185° but gave no rotation.

*Preparation of Cholesteryl p-Aminobenzoate.*

1 gm. of cholesteryl *p*-nitrobenzoate was dissolved in 150 cc. of ethyl acetate, 0.2 gm. of platinum oxide catalyst added, and the mixture shaken with hydrogen at 35 to 40 pounds pressure for 25 minutes in the apparatus described by Adams and Voorhees (13). After the platinum was filtered off and the ethyl acetate distilled off, the crude product was dissolved in benzene and washed with sodium bicarbonate solution and water. After the mixture was filtered, alcohol was added, which caused the *p*-aminobenzoate to separate out. After drying, it melted at 237.4–238.5° and its rotation was  $[\alpha]_D^{25} = +3.61^\circ$  in  $\text{CHCl}_3$ . A second preparation was made in order to check this rotation and it gave  $[\alpha]_D^{25} = +3.50^\circ$  in  $\text{CHCl}_3$ . It was absolutely insoluble in water and dilute hydrochloric acid.

Analysis (Kjeldahl). 0.1502 gm. substance required 29.50 cc. 0.01 *N* HCl.  
 $\text{C}_{34}\text{H}_{51}\text{O}_2\text{N}$ . Calculated. N 2.77 per cent. Found. 2.75 per cent.

*Saponification of Cholesteryl p-Aminobenzoate.*

1 gm. of the above product was saponified by refluxing with alcoholic potassium hydroxide. After dilution with water, the cholesterol was extracted with ether. After the ether solution was filtered, the ether was distilled off and the residue recrystallized from alcohol. The product possessed the same melting point as the original cholesterol and gave a rotation in  $\text{CHCl}_3$  of  $-39.30^\circ$ .

*Preparation of Cholesteryl p-Aminobenzoate Hydrochloride.*

Dry hydrogen chloride was passed into an ethereal solution of cholesteryl *p*-aminobenzoate at 0°. After 2 minutes the precipitated hydrochloride was filtered off and heated *in vacuo* to remove any traces of solvent. It melted at 210–211° and was insoluble in water or dilute hydrochloric acid.

Analysis. 0.2280 gm. substance: 0.0599 gm. AgCl.  
 $\text{C}_{34}\text{H}_{52}\text{O}_2\text{NCl}$ . Calculated. Cl 6.54 per cent. Found. Cl 6.49 per cent.

That hydrogen chloride had not been added to the double bond was shown by the fact that neither the benzoate nor *p*-nitrobenzoate gave a precipitate when an ethereal solution of these substances was treated with HCl at 0° for 2 minutes.

### *Preparation of Dihydrocholesterol.*

5 gm. of cholesterol were dissolved in 160 cc. of ethyl acetate and 2 gm. of platinum oxide catalyst added. The mixture was shaken with hydrogen at 35 to 40 pounds pressure for 4 hours. After the platinum was filtered off and the solution concentrated slightly, the dihydrocholesterol separated out in colorless crystals which were then recrystallized twice from alcohol. It melted at 142–143° and its rotation in CHCl<sub>3</sub> was +26.20°. In ether  $[\alpha]_D^{25} = +31.30^\circ$ . A second preparation was made to check the above rotation. It gave +26.35° in CHCl<sub>3</sub>.

Analysis. 0.1181 gm. substance: 0.3605 gm. CO<sub>2</sub>, 0.1294 gm. H<sub>2</sub>O.

C<sub>27</sub>H<sub>48</sub>O. Calculated. C 83.50 per cent, H 12.37 per cent.

Found. " 83.25 " " " 12.26 " "

A sample of dihydrocholesterol was prepared by catalytic reduction in glacial acetic acid as a solvent. This product melted at 141–142° and gave the following rotations:  $[\alpha]_D^{25} = +23.17^\circ$  in CHCl<sub>3</sub> and +28.05° in ether. These values agree with those previously reported by Anderson (5), von Fürth and Felsenreich (14), Willstätter and Meyer (15), and Ellis and Gardner (16). This product, however, was incompletely reduced as shown by the fact that it gave a positive Liebermann-Burchard reaction.

### *Derivatives of Dihydrocholesterol.*

The benzoate, *p*-nitrobenzoate, *p*-aminobenzoate, and the hydrochloride of the latter were prepared by exactly the same procedure as outlined above for cholesterol.

#### *Dihydrocholesteryl Benzoate.*

Colorless needles; m. p. 135.8–136.8°;  $[\alpha]_D^{25} = +21.40^\circ$  in CHCl<sub>3</sub>.

Analysis. 0.1223 gm. substance: 0.3700 gm. CO<sub>2</sub>, 0.1153 gm. H<sub>2</sub>O.

C<sub>34</sub>H<sub>52</sub>O<sub>2</sub>. Calculated. C 82.92 per cent, H 10.57 per cent.

Found. " 82.51 " " " 10.55 " "

## R. L. Shriner and L. Ko

### *Dihydrocholesteryl p-Nitrobenzoate.*

Light yellow needles; m. p. 156.5–157.7°;  $[\alpha]_D^{25} = +20.05^\circ$  in  $\text{CHCl}_3$ .

Analysis (Kjeldahl). 0.1342 gm. substance required 24.80 cc. 0.01 N HCl.

$\text{C}_{34}\text{H}_{51}\text{O}_4\text{N}$ . Calculated. N 2.60 per cent. Found. N 2.58 per cent.

### *Dihydrocholesteryl p-Aminobenzoate.*

Very light yellow amorphous product, insoluble in water and dilute hydrochloric acid. M. p. 191–192°;  $[\alpha]_D^{25} = +26.55^\circ$  in  $\text{CHCl}_3$ .

Analysis (Kjeldahl). 0.1483 gm. substance required 28.15 cc. 0.1 N HCl.  $\text{C}_{34}\text{H}_{53}\text{O}_2\text{N}$ . Calculated. N 2.76 per cent. Found. N 2.65 per cent.

### *Dihydrocholesteryl p-Aminobenzoate Hydrochloride.*

White amorphous product insoluble in water. M. p. 182.5–184.5°.

Analysis. 0.2212 gm. substance: 0.0581 gm. AgCl.

$\text{C}_{34}\text{H}_{53}\text{O}_2\text{NCl}$ . Calculated. Cl 6.53 per cent. Found. Cl 6.49 per cent.

### SUMMARY.

1. The benzoates, *p*-nitrobenzoates, *p*-aminobenzoates, and the hydrochlorides of the *p*-aminobenzoates of cholesterol and dihydrocholesterol have been prepared and their properties studied.

2. The best solvent for use in the catalytic reduction of cholesterol is ethyl acetate.

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## ANTIRICKETIC SUBSTANCES.

### VIII. STUDIES ON HIGHLY PURIFIED ERGOSTEROL AND ITS ESTERS.\*

BY CHARLES E. BILLS AND EDNA M. HONEYWELL.

(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana.)

(Received for publication, August 1, 1928.)

It is generally understood that the principal sterol of fungi is ergosterol. This substance was extracted from ergot by Tanret (1908), who purified it with notable success. Several later workers have employed yeast as a more convenient source, but the melting points and rotations of their preparations are not in agreement and do not equal the criteria established by Tanret (Table I). Failure to attain satisfactory purification is due to the instability of the ergosterol, and especially to the difficulties inherent in separating mixed sterols by crystallization.

The following procedure was designed to prepare ergosterol of the greatest possible purity for research on antiricketic activation. Crude yeast sterol was obtained from the unsaponifiable fraction of the fat of the common yeast, *Saccharomyces cerevisiæ*. Examination of many commercial batches of this product showed it to consist of at least three sterols, separable with difficulty. One is a low melting sterol, perhaps identical with the zymosterol of MacLean (1928). Another melts above 240°, and may be named cerevisterol without too great offense to etymology. Ergosterol itself is the main component. Imperfectly separated mixtures of these three sterols melt anywhere from 80–240°, and have apparently been mistaken for pure ergosterol in the literature.

Investigation of the effectiveness of several common solvents and many solvent mixtures in the purification of ergosterol led us to adopt alcohol-benzene for recrystallization. 50 gm. of the

\* Presented at the meeting of the American Chemical Society, Swampscott, Mass., September 11, 1928.



crude sterol were dissolved in 1 liter of a boiling mixture of 95 per cent alcohol (2 volumes) and benzene (1 volume). The hot solution was rapidly filtered through hardened paper on a silvered vacuum funnel, and cooled slowly until a slight turbidity just became perceptible in the supernatant fluid above the ergosterol crystals (beginning precipitation of cerevisterol or zymosterol).

TABLE I.

*Showing the Melting Point and Specific Rotation of Yeast Ergosterol as Reported by Different Investigators.*

Investigator.	Melting point.	Specific rotation in chloroform.	Remarks.
	°C.		
Gérard (1895).....	135-136	$[\alpha]_D = -105^\circ$	
Hinsberg and Roos (1903).....	159		
Neville (1913).....	145-147		$[\alpha]_D = -75.54^\circ$ in unspecified solvent.
Meisenheimer (1915)...	156-157		
MacLean and Thomas (1920).....	154	$[\alpha]_D^{18} = -117^\circ$	
Windaus and Grosskopf (1922).....	154		
Reindel, Walter, and Rauch (1927).....	160-161	$[\alpha]_D = -117^\circ$	
Reindel and Walter (1928).....	160		Purified <i>via</i> acetate.
MacLean (1928).....	158.5	$[\alpha]_D = -100^{**}$	Yeast presumably the source. Chloroform presumably the solvent for $[\alpha]_D$ .
Windaus and Borgeaud (1928).....	163	$[\alpha]_D = -130^\circ$	
Bills and Honeywell...	$\begin{cases} 168 \\ 175 \\ 183 \end{cases}$	$[\alpha]_D^{20} = -132^\circ$	Nearly anhydrous. Semihydrous. Very hydrous.

\* Calculated from MacLean's value,  $[\alpha]_{5461} = -130^\circ$ . We determined that  $[\alpha]_{Hg} \div [\alpha]_D = 1.297$  for ergosterol in  $CHCl_3$  at  $20.0^\circ$ .

Ergosterol came out of the liquid as acicular leaflets, while most of the pigment and contaminant sterols remained in solution. The ergosterol crystals after being washed with a little 95 per cent alcohol and pressed dry were almost snow-white, and under the microscope appeared homogeneous.

On account of the instability of ergosterol all purification treat-

ments must be conducted as expeditiously as possible, in the absence of strong light, and with minimum heating. Merely boiling ergosterol in such a low boiling solvent as acetone results in some decomposition, this being greatly accelerated by traces of catalysts, such as charcoal.

The best criterion of the purity of ergosterol is its optical rotation, as determined in chloroform solution. Since the hydrated form of ergosterol gives turbid solutions in chloroform (Tanret), it is necessary to dehydrate all test samples. Our procedure in every case was to subject the sample to the vacuum of a Hyvac pump for 1 hour at room temperature and then for 10 minutes at 80°. The accuracy of our polarimetric determinations was checked by testing a Bureau of Standards sucrose sample, our findings agreeing with those of the Bureau to within 0.01°.

The once recrystallized ergosterol, dehydrated, exhibited  $[\alpha]_D^{20} = -129^\circ$ . The particular batch of crude sterol from which this ergosterol was prepared gave  $[\alpha]_D^{20} = -120^\circ$ . Obviously one recrystallization effected the removal of a considerable amount of impurity of low optical activity. The ergosterol was recrystallized a second time from 500 cc. of alcohol-benzene, giving snow-white crystals,  $[\alpha]_D^{20} = -132^\circ$ ; yield, 31 gm. A third and a fourth recrystallization effected no significant change in specific rotation, and so it appeared that the limit of purification by this means had been reached. However, this is no proof that the ergosterol was pure. One recalls the failure of several recent attempts to separate cholesterol-ergosterol mixtures by crystallization alone.

In the fractionation of sterols by crystallization better results are sometimes obtained by working up the esters. In this way, *via* the acetate, Reindel, Walter, and Rauch (1927) and Reindel and Walter (1928) effected partial purification of a crude yeast ergosterol. With us another ester, the isobutyrate, was more satisfactory than the acetate. Ergosteryl isobutyrate can be crystallized in remarkably large, perfect prisms, which present a smaller area for surface contamination than either the acetate or free sterol.

Another sample of anhydrous ergosterol, partially purified,  $[\alpha]_D^{20} = -128^\circ$ , was converted into isobutyrate as follows: 10 gm. of sterol and 20 cc. of isobutyric anhydride, *free from chloride*, were heated in an oil bath for 10 minutes at 135–140°. The pale

yellow product crystallized solid on cooling. The hard mass was carefully comminuted in 100 cc. of 80 per cent acetone, allowed to stand for 1 hour, filtered, washed thoroughly with 80 per cent acetone, and dried in air at room temperature. The snow-white product was recrystallized from boiling ether-acetone (1:3) by refrigeration overnight. The resultant massive crystals, 1 to 2 cm. long, were washed with a little pure acetone and dried.

The limit of purification was reached by a second recrystallization, and the ester was then saponified: 7.5 gm. of ergosteryl isobutyrate were added to 500 cc. of boiling 97 per cent alcohol containing 11 gm. of purified NaOH. The heating was continued for 5 minutes after the last crystal dissolved. The solution was cooled to 35°, diluted with 125 cc. of water, and refrigerated for 6 hours. The precipitate was filtered with suction, washed with 80 per cent alcohol, and then with 65 per cent alcohol until the filtrate was neutral to litmus. The fine, colorless product was recrystallized twice from alcohol-acetone (1:1). Yield, 5.6 gm. of snow-white leaflets.

The ergosterol thus purified *via* the isobutyrate exhibited the same specific rotation,  $-132^\circ$ , as that purified directly by crystallization from the mixture of alcohol and benzene. This fact may be regarded as evidence, not alone conclusive, that the ergosterol was pure.

We have already mentioned the two contaminant sterols associated with yeast ergosterol. Both are less soluble than ergosterol in most solvents, and it might therefore be suspected that the exceptionally high specific rotation,  $-132^\circ$ , was due to admixture with them. However, this is not the case, for the specific rotation of the contaminants is low. A specimen of zymosterol, m.p.  $80^\circ$ , gave  $[\alpha]_D^{20} = +7^\circ$  in chloroform, and a cerevisterol preparation, m.p.  $240^\circ$ , gave  $[\alpha]_D^{20} = -49^\circ$ . Since both specimens were but sparingly soluble in chloroform, and neither was carefully purified, the rotations given are only suggestive. They prove, nevertheless, that the high rotation found for ergosterol is not due to either contaminant.

The value,  $[\alpha]_D^{20} = -132^\circ$ , is the highest ever ascribed to ergosterol from yeast, and it is identical with the figure given by Tanret (1908) for his most carefully purified anhydrous ergosterol from ergot. In ergot the principal contaminant is the feebly levorota-

tory fungisterol, which Tanret found *more* soluble than ergosterol. Tanret's description of the properties of fungisterol clearly differentiates it from either of the contaminant sterols of yeast. It is probable, therefore, that both his preparation of ergosterol and ours are pure, because they exhibit the same high specific rotation, and are surely not likely to be similarly contaminated.

The identity of our ergosterol with Tanret's is further indicated by crystallographic comparison. Tanret reported the data on crystal structure given by his associate, Lacroix, who examined a specimen in some detail. For the examination of our product we thank Dr. J. T. Singewald and Mr. E. H. Watson of the Johns Hopkins University. Specially large crystals were obtained from a solution of the pure ergosterol in ether-acetone (1:3). These were found to consist of elongated six-sided prisms, the angles adjacent to the long sides measuring about  $127^\circ$ , and the angles between the short sides  $106^\circ$ . The extinction is parallel, the sign positive, and the plane of the optical axes transverse to the length. It is somewhat uncertain whether the crystals are monoclinic or orthorhombic.

It would seem that the recent discoveries in the relation of ergosterol to vitamin D have all been made with ergosterol of questionable purity. Until now only Tanret had prepared ergosterol free from contamination, but this was done years before the current studies on activation. We desired to establish whether the antiricketic and spectrographic properties of ergosterol are characteristic of the pure sterol, or are associated with a contaminant. In cholesterol, as we have previously shown (1928), these properties are partly, though not wholly, due to contamination. While the details of our studies on activation will be published later, it is sufficient to state now that pure ergosterol, unlike zymosterol and cerevisterol, is highly activatable. We have prepared irradiated specimens so potent that 1 part in more than a billion parts of diet gave evidence of action in rat rickets. Spectrographic studies by Mr. W. M. Cox, Jr., of this laboratory, have shown that the absorption bands, found by other investigators, in ordinary ergosterol are in fact given by pure ergosterol, and not by zymosterol or cerevisterol.

To Tanret's good description of the properties of ergosterol may be added some notes of our own. Ergosterol does not exhibit

mutarotation in chloroform. However, on standing a week or more, the chloroform reacts with the ergosterol, the solution gradually darkening. Exposed to daylight, the solution deteriorates rapidly, becoming brown in a few hours. The reaction with chloroform is quite different from the yellowing that occurs in inert solvents.

Pure ergosterol is slightly less unstable than ergosterol of ordinary quality. Nevertheless it gradually becomes yellow or greenish yellow when kept at room temperature (25–30°). Exposure to light accelerates the yellowing. At 0° in the dark a sample of ordinary ergosterol exposed to the air has remained colorless for a year.

The specific rotation of ergosterol varies considerably with the temperature, this effect being relatively greater for  $[\alpha]_{H_2}$  than for  $[\alpha]_D$ . The following data were calculated for 20.0° and 25.0°, when  $c = 1$  in chloroform:

$$[\alpha]_D^{20} = -131.6^\circ$$

$$[\alpha]_D^{25} = -130.5^\circ$$

$$[\alpha]_{5451}^{20} = -170.6^\circ$$

$$[\alpha]_{5451}^{25} = -165.6^\circ$$

Crystals of anhydrous ergosterol and ergosteryl esters become highly electrified by friction with metal. Several cholesteryl esters and ethers behave similarly.

Ergosterol in benzene solution is destroyed by the clay catalyst, floridin. The decomposition occurs at room temperature and with far greater rapidity than Bills (1926) noted in the catalytic degradation of cholesterol.

The melting point of ergosterol as ordinarily reported is of little significance. Early in our work we made hundreds of melting point determinations, but we were seldom able to obtain the same value twice in succession, even on samples presumably homogeneous. The explanation of this anomaly was found in the fact that the melting point of ergosterol is exceedingly sensitive to differences in the water content of the sample.

In sealed capillary tubes *totally submerged* in the heating bath hydrated pure ergosterol (dried in moist air at 30°) began to sinter at 165°, melted clear at 175°, solidified at 174°, remelted clear at

178°, and resolidified at 174°. The same preparation, dehydrated, sintered at 160°, melted clear at 168°, solidified at 167°, remelted clear at 171°, and resolidified at 167°. This preparation was not indeed completely anhydrous. In fact, we never made a preparation that was, because we preferred to leave the last traces of moisture that were tenaciously held, rather than to risk decomposition in the drastic treatment necessary to remove them. After exposure to moisture, this product again gave the melting points of the hydrated form. When the dehydrated ergosterol was wetted with 25 per cent alcohol, and then dried only enough so that it was not sensibly wet, although fully hydrous, it melted clear at about 183°. When dehydrated ergosterol was examined in sealed capillary tubes only partially immersed in the heating bath, the m.p. was observed to be about 2° lower than when the tubes were completely immersed—an effect due obviously to the last traces of moisture being driven into the cool part of the capillary.

The foregoing melting points are corrected averages for a group of consistent readings. In sealed tubes ergosterol suffers little decomposition, for it can be remelted several times without altering the remelting and solidification points. Tanret also had trouble with his melting points, obtaining different values by different methods. He attributed the difficulty to oxidation, but more probably it was a matter of hydration. One may state that pure ergosterol melts at from 166–183°, according to the degree of hydration.

Tanret described four esters of ergosterol—formate, acetate, propionate, and *n*-butyrate, and noted that they crystallize water-free. Reindel, Walter, and Rauch prepared the benzoate. We again made the acetate and benzoate, and also three new esters—isobutyrate, isovalerate, and cinnamate. Each of our preparations was recrystallized to the limit of purification. Only the cinnamate exhibits colors on cooling from the molten state, whereas most of the esters of cholesterol give color play. Ergosterol esterifies readily, the cinnamate apparently forming at considerably greater velocity than cholesteryl cinnamate under the same conditions.

*Ergosteryl Acetate.*—The method of preparation is analogous to that for isobutyrate. Yield, 6.4 gm. from 10 gm. of ergosterol.

Snow-white leaflets from ether-acetone (1:3).  $[\alpha]_D^{25} = -90^\circ$ , when  $c = 1$  in  $\text{CHCl}_3$ . This ester sinters at  $178^\circ$ ; liquifies at  $179^\circ$ ; melts clear at  $181^\circ$  (corrected).

*Ergosteryl Isobutyrate*.—The method of preparation was as above described. Yield, 7.5 gm. from 10 gm. of ergosterol. Massive snow-white crystals from ether-acetone (1:3).  $[\alpha]_D^{25} = -84^\circ$ , when  $c = 1$  in  $\text{CHCl}_3$ . This ester sinters at  $145^\circ$ ; becomes a viscous turbid liquid at  $148^\circ$ ; becomes a thin turbid liquid at  $159^\circ$ ; melts clear at  $162^\circ$ .

*Ergosteryl Isovalerate*.—The method of preparation was analogous to that for isobutyrate, but recrystallizations were from pure acetone. Yield, 5.4 gm. from 10 gm. of ergosterol. Very fine snow-white needles, odorless.  $[\alpha]_D^{25} = -82^\circ$ , when  $c = 1$  in  $\text{CHCl}_3$ . This ester sinters at  $135^\circ$ ; becomes a viscous turbid liquid at  $138^\circ$ ; becomes a thin turbid liquid at  $157^\circ$ ; melts clear at  $160^\circ$ .

*Ergosteryl Benzoate*.—Prepared by the usual method with benzoyl chloride in cold pyridine. Recrystallizations from acetone-benzene 4:1. Yield, 5.4 gm. from 10 gm. of ergosterol. Small needles after prolonged standing in solvent at  $0^\circ$ ; highly electrifiable.  $[\alpha]_D^{25} = -177^\circ$ , when  $c = 1$  in  $\text{CHCl}_3$ . This ester sinters at  $162^\circ$ ; liquifies at  $164^\circ$ ; melts clear at  $168^\circ$ .

*Ergosteryl Cinnamate*.—10 gm. of dehydrated ergosterol and 10 gm. of cinnamoyl chloride were heated at  $170^\circ$  for 5 minutes. The product was dissolved in ether at room temperature, and filtered through hardened paper. To the clear solution 1.5 volumes of 95 per cent alcohol (2850 cc.) were added. The solution after standing overnight at  $-16^\circ$  was filtered, the cinnamate leaflets washed with a little pure acetone, and dried. Recrystallizations were made from ligroin purified by permanganate. Yield, 6.8 gm. of colorless, silvery, lustrous leaflet masses.  $[\alpha]_D^{25} = -22^\circ$ , when  $c = 1$  in  $\text{CHCl}_3$ . This ester sinters at  $175^\circ$ ; liquifies at  $177^\circ$ ; melts clear at  $179^\circ$ . On cooling, the melt solidifies at from  $145$ – $140^\circ$  with a beautiful play of colors.

#### SUMMARY.

1. Crude yeast sterol is a mixture of ergosterol and two other sterols. One is dextrorotatory and possibly identical with Mac-

Lean's zymosterol. The other, named cerevisterol, is levorotatory, and melts above  $240^{\circ}$ .

2. The ergosterol of yeast is identical with that of ergot, and has been isolated in pure form. Purification was accomplished in two ways—by recrystallization from an exceptionally effective solvent mixture (alcohol-benzene (3:1)), and by saponification of purified ergosteryl isobutyrate.

3. Pure ergosterol exhibits in chloroform  $[\alpha]_D^{20} = -132^{\circ}$  and  $[\alpha]_{5461}^{20} = -171^{\circ}$ . The melting point is of little significance as an index of purity, for it varies from  $166$ – $183^{\circ}$ , according to the hydration of the sample.

4. The spectrographic and physiological properties associated with ergosterol of ordinary purity are exhibited by ergosterol free from contaminants.

5. Three new esters, ergosteryl isobutyrate, isovalerate, and cinnamate, have been prepared and purified.

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## THE ESTIMATION OF SILICA IN TISSUES.

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The estimation of silica in animal organs by the gravimetric method is attended with considerable difficulty. Large amounts of tissue are necessary, and ashing by the method of Stolte (1), or even with the aid of an electric muffle furnace is a tedious process requiring several hours for completion. Analysis of the ash for silica in the conventional way also takes considerable time, so that an estimation is a fairly lengthy process.

Isaacs (2) seemed to have supplied the need for a rapid and easily manipulated procedure in his colorimetric method for the estimation of silica in tissue. Discordant and inaccurate results, however, attended the use of Isaacs' method in this laboratory. The colors produced in both standard and test solutions were not proportional to the concentrations, and in many cases, even with the utmost care, duplicate estimations did not check. In the case of tissues high in calcium, there was invariably formed, when the test solutions were heated in the water bath, a heavy precipitate of calcium molybdate. This precipitate was a source of constant trouble, as the test solutions had to be filtered before they could be read, and in several instances it was thought that some of the blue color was being carried down by the calcium molybdate. At any rate the results obtained by this method did not agree with gravimetric analyses done as a check on the same tissues, and the discrepancy was largest where the precipitate in the solution had been heavy.

In the present paper a micro method is proposed which makes use of the intense yellow color formed when a silicate solution is treated with ammonium molybdate and sulfuric acid. The method is quick, and easily applied, and the proportionality of

color produced in test solutions prepared from sodium silicate and from ashed tissue holds over a wide range of concentration.

The production of yellow silicomolybdic acid formed the basis for Dienert and Wandenbuleke's (3) colorimetric method for estimating small amounts of silica in water, and was used by Atkins (4) and by Thresh (5) in their studies of the silica content of natural waters.

The yellow color is of the same tint as that of a dilute solution of picric acid, so that an artificial standard is possible. An artificial standard is preferable to a sodium silicate standard since the latter tends to deposit silica, and is difficult of standardization.<sup>1</sup> The picric acid standard remains unchanged for at least 2 months, and can be made from any good brand of c.p. picric acid without recrystallization.

Phosphate, reckoned as  $P_2O_5$ , gives less than a tenth of the color given by an equal weight of  $SiO_2$ . In some cases where the phosphorus content is low a fairly reliable estimate of the silica present may be got without precipitation of the phosphate. But for its accurate determination, the removal of phosphate is necessary, and is accomplished by adding magnesia mixture to the solution of the ash, and filtering off the magnesium ammonium phosphate.

That the silica is not partially precipitated along with the phosphate, but is quantitatively estimated by the method, is shown by the following experiments.

### *Experiment I.*

The silica of a mixed silicate phosphate solution is recovered in the filtrate after precipitation of the phosphate.

*Solution 1.*—10 cc. of sodium silicate solution containing 1 mg. of  $SiO_2$ , were diluted to 40 cc. with water, treated with 2 cc. of 10 per cent ammonium molybdate and 4 drops of 50 per cent (by volume) sulfuric acid, and made to 50 cc.

*Solution 2.*—1 mg. of  $SiO_2$  and 2 mg. of P as  $K_2HPO_4$  in 35 cc. of water were treated with magnesia mixture and ammonia.

<sup>1</sup> Isaacs used silicate solutions made of highly diluted water glass, which he standardized against a solution containing a known weight of  $SiO_2$ , which had been fused with sodium carbonate and dissolved in water.

After being filtered and washed, the solution was neutralized, treated with ammonium molybdate and sulfuric acid, and made to volume.

*Solution 3.*—1 mg. of  $\text{SiO}_2$  and 5 mg. of P were treated in the same way.

These solutions had the same depth of yellow color.

### *Experiment II.*

Silica added to tissue is quantitatively recovered as shown by difference. Several different tissues were analyzed according to the method before and after the addition of silica.

Lung 1 gave 11.40 mg. of  $\text{SiO}_2$  per gm. of dried tissue, and 16.40 mg. after the addition of 5 mg. of  $\text{SiO}_2$  per gm.; difference 5 mg.

Lung 2 gave 6.80 mg. of  $\text{SiO}_2$  per gm. of dried tissue, and 11.76 mg. after the addition of 5 mg. of  $\text{SiO}_2$  per gm.; difference 4.96 mg.

Liver 1 gave 0.80 mg. of  $\text{SiO}_2$  per gm. of dried tissue, and 1.28 mg. after the addition of 0.5 mg. of  $\text{SiO}_2$  per gm.; difference 0.48 mg.

Egg gave 0.2 mg. of  $\text{SiO}_2$  per 10 gm. of white, and 0.69 mg. after the addition of 0.5 mg. of  $\text{SiO}_2$ ; difference 0.49 mg.

### *Experiment III.*

Values given by the colorimetric procedure check closely with those obtained by the gravimetric method. The results of several analyses are as follows:

#### *Comparison of $\text{SiO}_2$ Values Given by Colorimetric and Gravimetric Methods.*

Sample 1. 0.2 gm. of silicotic Lung 409 analyzed colorimetrically gave 63.0 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

5.0 gm. of the same ashed in an electric muffle furnace and analyzed gravimetrically gave 0.3180 gm. of  $\text{SiO}_2$  or 63.6 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

Sample 2. 0.25 gm. of silicotic Lung 410 gave by the colorimetric method 60.0 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

5.0 gm. of the same gave by the gravimetric method 0.3035 gm. of  $\text{SiO}_2$  or 60.7 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

Sample 3. 0.1 gm. of silicotic Lung 411 gave by the colorimetric method 29.6 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

8.5 gm. of the same gave by the gravimetric method 0.2468 gm. of  $\text{SiO}_2$  or 29.0 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

Sample 4. 0.5 gm. of Liver 407 gave by the colorimetric method 1.33 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

50.0 gm. of the same gave by the gravimetric method 0.0660 gm. of  $\text{SiO}_2$  or 1.32 mg. of  $\text{SiO}_2$  per gm. of dried tissue.

In Isaacs' method the presence of iron in the ash from those tissues containing considerable amounts of blood, such as liver and kidney, resulted in a greenish tint in the test solution. Isaacs diluted his standard with a solution of ferric alum till it matched the green color of the test, and made allowance for the dilution in his calculation. It has been found impossible in this laboratory to get even approximate matches in this way, and the dilution

TABLE I.  
*Silica Content of Tissues.*

		SiO <sub>2</sub> per gm. dried tissue.	SiO <sub>2</sub> per gm. fresh tissue.	Total SiO <sub>2</sub> in organ.
		mg.	mg.	mg.
Lung.	Guinea pig.....	0.78	0.12	0.50
	" " .....	0.48	0.06	0.25
	Rabbit.....	1.70	0.27	2.38
	" .....	2.02	0.30	2.93
	Dog.....	2.06	0.32	60.72
	" .....	2.50	0.39	52.50
	Human (normal).....	1.40		
	" (silicotic).....	123.55		
Kidney.	Rabbit.....	1.97	0.25	2.96
	" .....	1.70	0.34	3.45
	Dog.....	1.12	0.21	7.28
	" .....	0.80	0.15	4.88
Liver.	Rabbit.....	1.90	0.32	10.50
	" .....	1.85	0.46	9.50
	Dog.....	1.33	0.34	77.71
	" .....	0.97	0.23	23.37
Spleen.	Dog.....	0.66	0.16	1.42
	" .....	0.49	0.11	0.53

factor in the standard is an additional source of error. In the present method iron is precipitated along with the phosphate, so that the procedure is as applicable to those tissues containing blood, as it is in the case of lung tissue where the iron content is low.

Some results of analyses of animal organs are given in Table I. The values are of the same order as those given by Gonnermann, (6) who used the gravimetric method.

*Solutions Required.*

All reagents must be silica-free, and should be tested for its presence by adding ammonium molybdate and a few drops of sulfuric acid. Of the solutions used, only sodium hydroxide is likely to contain silica. It is therefore necessary to prepare this solution from metallic sodium, when a silica-free reagent will be obtained.

*Nitric acid*, concentrated.

*Ammonium nitrate*, 20 per cent solution.

*Boric acid*, saturated solution.

*Normal sodium hydroxide*, made by dissolving 2.3 gm. of metallic sodium in water in a nickel crucible, making to 100 cc., and keeping in a paraffined bottle.

*Sulfuric acid*, 50 per cent by volume.

*Magnesia mixture*, 5.5 gm. of magnesium chloride and 7 gm. of ammonium chloride dissolved in water and made to 100 cc.

*Ammonium Hydroxide 2.5 Normal*.—Several samples of aqueous ammonia gave no test for silica. If a positive test is given, a silica-free solution should be prepared by distilling ammonia through a reflux condenser and bubbling the gas into distilled water contained in a paraffined bottle packed in ice. To prevent the water backing up the tube and into the condenser, a slow current of air is sucked, by means of a vacuum pump, through the entire apparatus. Specimens of the solution in the paraffined bottle may be titrated at intervals and the distillation stopped when the desired strength is reached.

*Ammonium molybdate*, 10 per cent solution.

*Standard Picric Acid*.—A stock solution is made by dissolving 102.4 mg. of pure vacuum-dried picric acid in water and making to 100 cc. By diluting 1 part of the stock solution to 100 with water a solution is obtained, 50 cc. of which have the same quality and intensity of color as that developed in a solution containing 1 mg. of  $\text{SiO}_2$  when treated with 2 cc. of ammonium molybdate and 4 drops of sulfuric acid and made to 50 cc.

*Method.*

The tissue to be analyzed is dried to constant weight at  $105^\circ$  and finely pulverized in an iron mortar. Ashing is carried out in

the wet way in a platinum crucible of 30 or 40 cc. capacity, though a smaller one may be used. An amount of the tissue (0.2 to 1.0 gm. is a convenient quantity) is treated with 3 cc. of nitric acid, 2 cc. of ammonium nitrate, and 1 cc. of boric acid. The boric acid prevents any loss of silica as fluoride. The mixture is heated on the water bath till frothing ceases and a clear solution is obtained and then to dryness on a hot plate or with a Bunsen flame. Care must be taken during the latter stage, or loss by spattering will occur. The crucible is then heated to redness over a Meker or Bunsen burner. The carbonaceous residue quickly oxidizes, leaving a white ash. Sometimes a second or even a third treatment with nitric acid and ammonium nitrate is necessary; this depends on the amount taken and on the type of tissue. Lung is easily ashed; liver and kidney usually require an additional treatment.

Solution of the silica in the ash is effected by adding 3 cc. of the  $N$  NaOH and several cc. of water and heating on the water bath. If the ash sticks to the bottom of the crucible and does not readily dissolve, the process is aided by rubbing it free with a rubber policeman. The crucible is now removed from the water bath, a drop of phenolphthalein added, and then dilute sulfuric acid till the pink color is almost discharged, leaving the solution faintly alkaline. Water is added to a volume of 25 or 35 cc., depending on the size of the crucible being used, and 1 cc. of magnesia mixture added for each 3 mg. of P present. (1 or 2 cc. are usually sufficient except in the case of tissue high in P such as brain.) 2 cc. of ammonia are added, a few minutes allowed for precipitation of the phosphate, and the solution then filtered through a small paper into a Pyrex test-tube graduated at 50 cc. The residue is thoroughly washed with distilled water, the washings being added to the filtrate. After the solution is neutralized, 2 cc. of ammonium molybdate and 4 drops of sulfuric acid are added, and water to 50 cc. The yellow color, which quickly develops, reaches a maximum in 5 minutes and remains constant for several hours.

Test solutions are conveniently matched in Hehner tubes against the picric acid standard, 50 cc. of which have a color equivalent to 1 mg. of  $SiO_2$  in the test. Improvised tubes can be made by sealing a small glass stop-cock in the side near the bottom of one of two 50 cc. graduated cylinders which appear to be identi-

cal as regards kind of glass, bore, height of graduation, etc. The standard solution is run out of the tube containing the stop-cock till it matches the test in the other tube. With a little practice, as accurate readings can be obtained with these tubes, when mounted on glass over white cardboard, as are got by the use of an expensive colorimeter. If Nessler tubes are used it is convenient to make several standards of different strength, by appropriate dilution of the stock solution of picric acid.

When the silica content of the tissue is abnormally high, as in silicotic lungs, it is advisable, after the sodium hydroxide is neutralized, to transfer the solution to a graduated cylinder, and use an aliquot portion for the estimation. While the 2 cc. of 10 per cent ammonium molybdate are sufficient for 3.5 mg. of  $\text{SiO}_2$ ,<sup>2</sup> it is desirable that the aliquot portion should contain about 1 mg. since the color of the test should fairly closely approximate that of the standard.

#### SUMMARY.

A micro method for the estimation of silica in animal tissues, which depends on the production of yellow silicomolybdic acid, has been described. Evidence is produced to show that the method gives accurate results.

The silica content of some tissues has been estimated; values are listed in tabular form.

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<sup>2</sup> Dienert and Wandenbuleke found that 2 cc. of 10 per cent solution of ammonium molybdate were enough to react with not more than 3.5 mg. of  $\text{SiO}_2$ . This observation has been confirmed by the author.





# LACTOSE METABOLISM IN WOMEN.

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## HISTORICAL RÉSUMÉ.

Ever since practical methods for the identification of sugar in the urine have found application in medical practice, it has been recognized that there often appears an abnormal amount of sugar, or at least of reducing substances, in the urines of women during confinement.

Lehmann (1), in a text-book published in 1850, reports that Heller, the year before, had observed an increase in the reducing power of urine during lactation. Blot (2) by means of polarimetric and fermentation tests claimed to have established the sugarlike nature of this substance. There followed, during the next 10 years, a controversy, principally between Brücke (3) and Leconte (4) as to the nature of the reducing substance found in the urines of lactating women. It was finally established by

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Brücke that the substance was a sugar, but it was erroneously assumed by all these early workers that it was the same sugar which occurred in diabetic urines. In 1873, however, de Sinéty (5) observed that these urines did not respond to the fermentation test as they would if glucose were present, and in 1877, Hofmeister (6) isolated from the urine of a lying-in woman a crystalline, optically active substance which was identical in all its properties with lactose. Since the time of this work of Hofmeister's, it has been generally accepted that lactosuria is a common phenomenon among women in confinement. Lemaire (7), in 1895, identified lactose by osazone formation, and quantitatively determined it in the urines of fifteen lactating women. His method was cumbersome and required large amounts of lactose to give a positive test. The same may be said of the method used by Porcher (8-12), who investigated the problem of lactose formation in mammals between the years 1902 and 1910. Porcher's work was mostly done on cows and goats, but in these animals, and also in a number of women, he identified lactose in the urine both before and after delivery. The principal object of his investigation was to prove that the lactose of the milk is formed in the mammary glands and has as its only source the glucose of the blood.

These experiments of Porcher are the latest ones reported in the literature that include observations upon lactosuria in women. What little work has been done on lactose metabolism in more recent years is mainly concerned with the results of injection or ingestion of lactose in normal animals, and without any special consideration of the problem of pregnancy and lactation. The present investigation includes observations on the results of lactose feeding during different stages of the sexual activities of women.

The scarcity of literature upon this subject may be due in part to the fact that there has been no convenient or dependable method of quantitatively analyzing urines for lactose.

#### *Method for Determination of Lactose in Urine.*

The following procedure was finally adopted: The urine is analyzed for "total sugar" by the regular Folin-Berglund (13) method; i.e., to a measured amount of urine (from 1 to 10 cc., depending upon its concentration) are added 5 cc. of a 0.1 N solution of  $\text{H}_2\text{SO}_4$ . The volume is then made up to 20 cc. with water, whirled for 2 minutes with 1.5 gm. of Lloyd's reagent, and then centrifuged. With a dry Ostwald pipette, 1 or 2 cc. of the supernatant fluid are transferred to a Folin sugar tube. If only 1 cc. of the solution was used, then 1 cc. of water is added before the 2 cc.

of Folin-Wu copper reagent, and the tube is placed in the boiling water bath. We have boiled for 10 minutes in all determinations and used the 1926 molybdic acid reagent (Folin (14)) for color developer. The reading is made against a 0.2 mg. glucose standard set at 20.0. There are now from 12 to 15 cc. of the solution left in the centrifuge tube, and unless by the foregoing determination it has been found that further dilution is necessary, this solution is transferred directly to an Erlenmeyer flask and put into the water bath to be warmed up to 38–42°. One-quarter of a cake of Fleischmann's yeast is then added, the flask stoppered, and shaken until it is thoroughly emulsified. The flask is then returned to the water bath for exactly 7 minutes, at the end of which time the mixture is immediately transferred to a centrifuge tube, and centrifuged rapidly. Within half a minute the yeast is firmly packed in the bottom of the tube, and we have found this to be the quickest and easiest way to stop the action of the yeast. The turbid supernatant fluid is decanted back into the Erlenmeyer flask, shaken for 2 minutes with 1.5 gm. of Lloyd's reagent, and again centrifuged. The clear supernatant fluid is then ready for the final sugar determination.

In working out the details of this procedure, we made the following observations.

7 minutes is sufficient to destroy glucose in urine up to a concentration of 0.25 per cent. A urine containing more glucose than this must be diluted before fermentation.<sup>1</sup>

A longer fermentation period must be avoided, since it has been found that after the first 10 or 15 minutes the reducing substances of normal urines undergo unexplained fluctuations which make the results on residual reduction unreliable. This finding may explain the fact that conflicting results are often found in the literature wherever fermentation tests have been used upon urine.

Eagle (15), in 1927, reported he had observed that residual reduction values after yeast fermentation were unreliable, and he explained his results on normal urines to which glucose had been added by stating that a "urine-yeast reaction" takes place which

<sup>1</sup> This short fermentation process was worked out in connection with Folin and Svedberg's investigation of the residual reduction of normal blood filtrates, and it is given on p. 418 of that paper, as applied to blood analysis. (Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1926, lxx, 405.)

interferes with the fermentation of glucose. By a preliminary treatment with Lloyd's reagent, he claims that the unknown substances which interfere with the fermentation of glucose are removed. This suggestion of Eagle's was carefully followed up, and it was found that in the short 7 minute fermentation period no such "urine-yeast reaction" takes place, the destruction of added glucose being complete whether the urine is treated with Lloyd's reagent before or after fermentation. However, it was found that if the urine was treated with Lloyd's reagent before fermentation and then cleared of yeast after fermentation by a second 2 minute shaking with Lloyd's reagent, this second treatment caused the further removal of from 0 to 25 mg. per cent of unknown reducing substances. (125 such duplicate analyses were carried out on the urines of pregnant women.) It was ascertained, by the addition of lactose to normal urines, that this further removal of reducing substances by the double Lloyd treatment did not involve any further removal of lactose.

Since the greatest weakness in the fermentation method of analyzing urines for lactose lies in the fact that the non-fermentable reducing substances of the urine include unknown reducing substances as well as lactose, any means of removing these substances without removing lactose is most acceptable. By the inclusion of the double Lloyd treatment in our procedure, this source of error, while by no means eliminated, is at least somewhat decreased.

Somogyi (16) has recently recommended the washing of yeast in his method for the determination of the residual reduction of the blood. We have found that by washing, the yeast blank can be reduced only from 4 or 5 mg. per cent to 1 or 2 mg. per cent. We have also found that in determining the residual reduction of urine by our method a suspension of unwashed yeast gives exactly the same results for residual reduction as does a suspension of washed yeast. But the unexpected observation was made that solid yeast removes from 4 to 8 mg. per cent more reducing substances from urine than does a yeast suspension. Since we have found that this further removal does not include any removal of lactose, it is evident that in the determination of lactose it is better to use solid yeast, which apparently takes out some of the unknown reducing substances as well as glucose.

Of course, if the object were to determine the glucose of the urine, one would use a suspension of washed yeast and avoid the double Lloyd treatment. (When washed yeast is used, no clarification after fermentation is necessary.) It must be borne in mind that, in the method used in this investigation, the difference between the "total sugar" and the "residual reduction" does not represent fermentable sugar alone, but rather includes fermentable sugar plus from 5 to 25 mg. per cent of unknown reducing substances. When the residual reduction is anything over 160 to 200 mg. per cent, we have considered it safe to label the results "lactose." In all analyses, the readings have been made against a glucose standard and the results are expressed in terms of glucose (unless otherwise specified). Glucose is 1.7 times as strong a reducer as lactose (as determined by the Folin-Wu sugar method) and therefore, to get the actual values in terms of lactose, our results must be multiplied by 1.7. Since comparative figures are fully as informative as actual values, and since in many cases we do not feel justified in labelling our results "lactose," we have kept all figures (unless otherwise specified) in terms of glucose.

Before application of this method to the investigation of the problem at hand, it was carefully tested out on a number of urines from normal individuals, diabetics, and lactating mothers. 120 urines were analyzed, and it was found that *except in border-line cases* it was easy to differentiate between glucosuria, lactosuria, and normal urines.

In order to ascertain the rate of lactose excretion the creatinine was also determined. It was found simpler to do this than to attempt to collect 24 hour specimens. Since the daily creatinine output of any individual is constant, and dependent upon the weight (Folin, 1905 (17)), it is possible by knowing the weight of the patient and the creatinine content of a given specimen, to calculate approximately what part of the 24 hour amount 1 cc. of that specimen would represent. Inasmuch as we are interested only in comparative values, however, that is, in following the rise and fall of the lactose excretion, it was decided to express the results in terms of lactose to creatinine ratios:  $\frac{\text{mg. lactose per cc.}}{\text{mg. creatinine per cc.}}$  (L:C), "total sugar" to creatinine ratios (TS:C), and residual reduction to creatinine ratios (RR:C). In this way, and by

always using for analysis the morning specimen (before any food has been taken) comparative values are acquired. During the course of this investigation forty-seven analyses were run on the morning urines of normal women and men. The RR:C ratios for the women ran between 0.220 and 0.590, the average being 0.403. The RR:C ratios for the men ran between 0.250 and 0.520, the average being 0.330. It was observed that normal individuals vary somewhat in their RR:C level from day to day, the women showing greater fluctuations than the men. This, of course, does not represent sufficient data from which to draw any conclusions concerning the residual reduction of normal urines. It was done simply to give some idea of the normal RR:C level, with which to compare the data on pregnant and lactating women.

The results to be presented, therefore, have been acquired by the use of the method described above. The limitations of this method are fully realized, but it has yielded, nevertheless, considerable data which have proved instructive, and suggest many possibilities concerning the relation of lactose metabolism in women to the processes of reproduction.

#### *Studies on Excretion of Lactose.*

If, as seems to have been shown by Folin and Berglund (13), there is no renal threshold for lactose and galactose, then any of these two sugars introduced, either normally (by the glands) or experimentally (by ingestion) into the circulation and not destroyed by the tissues will show up in the urine. It would seem, therefore, that the study of the appearance of lactose (and galactose) in the urines of the individuals experimented upon would give some rather valuable insight into the metabolism of these sugars.

#### *Daily Lactose Excretion of Lactating Women.*

The first line of investigation was on the ward patients at the Boston Lying-In Hospital. Since this is an emergency hospital only, the patients do not come in until the day of delivery, and leave from 10 to 14 days after. On twenty-two such women, the daily urines were analyzed. Representative results are given in Table I.

Since it is not practical to present all the data collected, we are

including only a few typical cases. Each individual varies both in the amount of lactose excreted, and in the degree of the fluctuations noted from day to day, but certain generalizations can be made which apply to all the cases observed.

On 90 such specimens a qualitative test, as well as the quantitative determination for total sugar and for lactose, was run. The qualitative test used was the regular Benedict test which is used in most hospitals for the routine urine analysis. We have found that any urine that contains more than 150 mg. per cent of "total

TABLE I.  
*Daily Urine Analyses on Lactating Women.*

Name.....	Mrs. Breslin.		Mrs. R. P.		Mrs. Wagner.	
Remarks....	Normal delivery Feb. 22. More than enough milk.		Normal delivery May 5. Definitely deficient in milk.		Normal delivery Mar. 4. Sufficient milk.	
Days after delivery.	Lactose in urine.		Lactose in urine.		Lactose in urine.	
	<i>mg. per 100 cc.</i>	<i>L:C*</i>	<i>mg. per 100 cc.</i>	<i>L:C</i>	<i>mg. per 100 cc.</i>	<i>L:C</i>
1	38.6	1.28			44.5	1.30
2	61.5	1.23	35	0.442	28.6	1.40
3						
4	310	4.40	63	0.641	1190	8.32
5						
6	500	4.85	133	1.33	981	7.60
7	222	2.89			208	1.36
8	255	2.30	61.8	1.42	Qualitat. Negative.	
9	250	2.50			615	6.48
10	410	3.28				
11	500	6.28			Qualitat. Negative.	
12						
13					Dismissed from hospital.	
14	123	3.39	105	1.12		
15	Dismissed from hospital.					
18			86.5	1.16		
20			86.5	1.16		
23			55	0.810		
26			38	0.550		
30			44.5	0.735		
31			Stopped nursing baby.			



TABLE I—*Concluded.*

Name.....	Mrs. Johnson.		Mrs. Evans.		Mrs. Trudell.	
Remarks....	Normal delivery Mar. 18. Plenty of milk, but discontinued nursing 9th day after delivery.		Normal delivery Apr. 5. Plenty of milk, but discontinued nursing 10th day after delivery.		Still-born baby Feb. 28.	
Days after delivery.	Lactose in urine.		Lactose in urine.		Lactose in urine.	
	<i>mg. per 100 cc.</i>	<i>L:C</i>	<i>mg. per 100 cc.</i>	<i>L:C</i>	<i>mg. per 100 cc.</i>	<i>L:C</i>
1	148	1.18	54	0.540	23	0.705
2	222	2.12	107	0.910		
3	194	1.61	147	1.17	47	3.76
4	211	1.68	630	7.52		
5	49.4	1.58	235	1.99		
6	510	3.82	335	2.19	16.4	0.740
7	222	1.40	294	1.91	7.7	0.390
8	457	1.83	205	1.24		
9			175	0.890	94	3.10
10	428	2.91	181	0.930	77.5	1.71
11	1000	7.50	176	1.10		
12	333	3.60	157	0.851	66	1.38
13	235	1.15	196	0.835		
14	133	1.33	91	1.18	69	0.680
15	Dismissed from hospital.		103	1.33	Dismissed from hospital.	

\* L:C = lactose to creatinine ratio.

sugar" usually gives a positive qualitative test (*i.e.* some yellow precipitate). Normal urines almost never contain more than 100 mg. per cent of reducing substances, but from the figures given in Table I, it is evident that "sugar-positive" urines from women during the first 2 weeks after delivery are frequent. It is not surprising, therefore, that even when qualitative tests alone have been used, it has been a common observation that "sugar" excretion is likely to occur at this time. It will also be evident, however, by comparison of the figures for lactose or residual reduction with those for the L:C ratios, that neither a qualitative nor a quantitative test for "sugar" gives any dependable indication of the actual daily sugar excretion. The time element as represented by the creatinine content of the urine must be determined and taken into account.

The first observation of note is that in every case studied the

L:C ratio on the 1st day after delivery is lower than at any later time. This low level lasts from 1 to 5 days, at the end of which time a sudden and often tremendous rise in lactose excretion occurs. (Mrs. B. C. (Table II) is the most striking example of this that has been observed. Her L:C ratio on the 4th day is 30 times as large as it was just after delivery.) We have not been able to discover that this observation of the low level of the lactose excretion on the first few days after delivery, followed by a sudden very marked excretion of lactose, has been noted before.<sup>2</sup>

The colostrum which is excreted by women during the first few days after delivery differs markedly in both quality and quantity from the milk which usually begins coming on the 3rd or 4th day. Also, during the first few days the new born baby is apparently not getting as much food as it does later for it loses weight. It has therefore been concluded that the mammary glands do not come into full activity until several days after delivery. It was at first thought, therefore, that the amount of lactose appearing in the urine could be taken as an indication of the degree of activity of the mammary glands. Further observations, however, proved that this was not the case. Mrs. Breslin (Table I) had more milk than any other case studied, but her L:C ratios were no higher than those of several other women who had no excess amount of milk. Mrs. B. C. (Table II) showed the highest lactose excretion of any of the cases followed, but her supply of milk was no greater than normal. There were a number of other cases illustrating the point.

Moreover, the great fluctuations in urinary excretion of lactose during the first 2 weeks after delivery, which were evident in all the cases studied, are not explained by any corresponding differences in milk secretion. Attempts were made to find some explanation for these extraordinary fluctuations. The specimens were all taken at the 6 o'clock morning nursing before any food had been taken. It would seem, therefore, that they are comparable with each other. Moreover, it was ascertained that the time of taking

<sup>2</sup> Rowley (18), in following the blood sugar of pregnant and lactating women has been able to discover no abnormalities in blood sugar except sometimes a slight hyperglycemia on the 2nd to the 4th day post partum. This observation is interesting in connection with the above. This worker, however, reports no glycosuria.

the specimen in relation to the time of nursing did not influence the lactose content. Hourly urine specimens showed no change in the L:C ratios before or after nursing. Also the fact that women with still-born babies (three cases were followed) showed these same fluctuations would seem to rule out suckling as an influence upon lactose excretion.

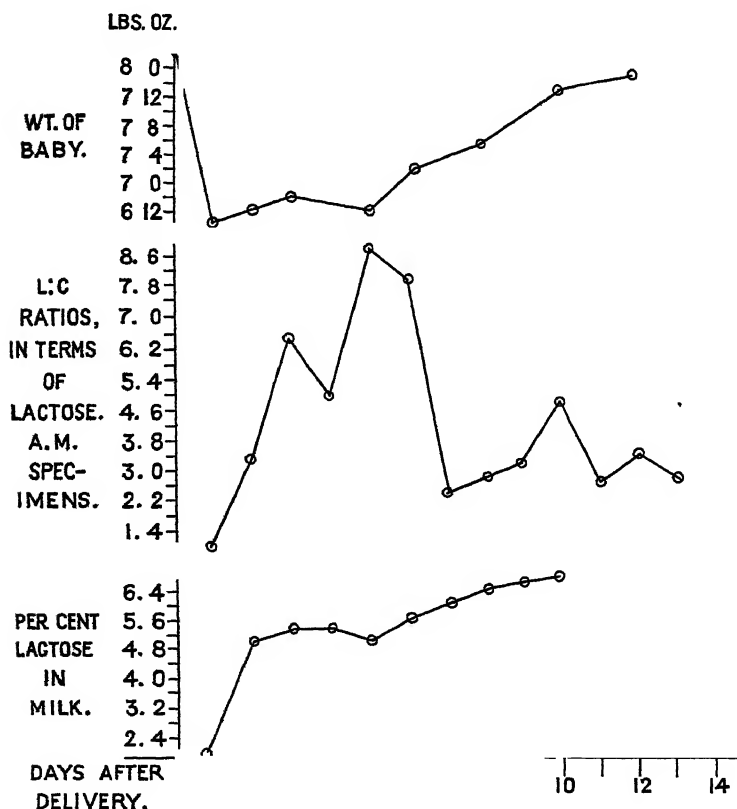


CHART I. Mrs. DiTullio.

Experiments were also carried out in an attempt to discover whether or not the composition of the milk from day to day, or the weight of the babies, had any connection with the fluctuations in L:C ratios. On three women, who were lactating normally, L:C ratios, the weight of the babies, and the lactose content of the

milk were followed daily for the 2 weeks that they were in the hospital. Chart I presents the data on one of the cases. All three of the patients showed practically the same percentage composition and constant rise in lactose content of the milk during the first 2 weeks. Also, all of the babies showed the usual drop in weight just after delivery, and constant increase during the succeeding days. But the fluctuations in the level of the L:C ratios were very marked, and seemed to have no connection with the other two lines of observation.

Two of the cases given in Table I are of women who discontinued nursing. One of the women, Mrs. Johnson, showed a large excretion of lactose on the 2nd day after her breasts were dried up, but with Mrs. Evans, this phenomenon did not occur, although she was having fully as much milk for the baby as was Mrs. Johnson. Porcher (8), in 1902, and Campus (19), in 1920, have observed that, in lactating cows, when suckling is suspended, there appears during the next few days a marked excretion of lactose, the amount of lactose in the urine at this time being in direct proportion to the amount of milk yielded by the cow. It seems quite reasonable that, as Porcher says, there should be a "backing up of lactose" when suckling is discontinued, until the mammary glands cease being active. But with women, at any rate, some individuals seem to be better able to take care of this condition without the help of lactose excretion than others, so that lactose excretion in this condition is not always proportional to the activity of the mammary glands.

On four women who never nursed their babies (Mrs. Trudell, Table I, is an example), the urines were analyzed. Under these conditions the glands in time cease to be active, and the L:C ratios assume a low level, as would be expected. But in all of these cases, up to the time when the glands ceased secreting milk, it was found that the fluctuations in L:C ratios from day to day were much the same as in normal women where suckling was going on.

No parallel can evidently be drawn, therefore, between the activity of the mammary glands and the excretion of lactose. It is, or course, true that where the mother is definitely deficient in supplying milk, the L:C ratios are low (Mrs. R. P., Table I, is the most striking example we have of this). But the reverse, that where the milk supply is large, the L:C ratio will be correspondingly high, evidently does not hold.

The picture presented up to this point was certainly a very complicated one. It was only upon following the urines at later periods during lactation that we were able to obtain any clue to the situation. Table II gives the results with four patients.

All of these women were still nursing their babies at these later dates, and producing more milk than at the earlier periods followed. The fluctuations in lactose excretion had apparently stopped and

TABLE II.  
*Daily Urine Analyses on Lactating Women.*

Mrs. P. B.			Mrs. L. B.		
Normal delivery Dec. 2. Enough milk most of the time.			Normal delivery Dec. 20. Plenty of milk.		
Date.	Lactose in urine.		Date.	Lactose in urine.	
	mg. per 100 cc.	L:C*		mg. per 100 cc.	L:C
Dec. 3	47.2	0.842	Dec. 21	48.2	0.862
" 4	152	1.38	" 22	80	1.46
" 5	130	1.97	" 23	365	2.20
" 6	97	1.42			
" 7	86	1.15			
" 8	49.6	0.875			
" 9	84.2	1.20			
" 10	114	1.76			
" 11	110	2.03			
" 12	80	1.56			
" 13	108	1.77			
" 14	97	1.34			
" 15	109	1.68			
" 16	151	1.51			
" 17	116	1.48	Jan. 5	225	2.01
" 18	118	1.30	" 6	180	1.76
" 19	180	1.52	" 7		
" 20	120	1.80	" 8	92	1.45
" 21	135	1.54	" 9		
" 22	123	1.53	" 10	77.2	1.24
Jan. 25	58.8	1.23	Jan. 25	128	1.16
" 26	128	1.35	" 26	62	1.10
" 27	138	1.34	" 27	97	1.12
" 28	198	1.20	" 28	94	1.18
" 29	174	1.18	" 29	153	1.19
" 30	138	1.32	" 30	133	1.13
Feb. 14	17.7	1.12	Feb. 14	55	0.832

TABLE II—*Concluded.*

Mrs. F. B.			Mrs. B. C.		
Normal delivery Dec. 30. Plenty of milk.			Normal delivery Dec. 17. Plenty of milk (no excess).		
Date.	Lactose in urine.		Date.	Lactose in urine.	
	<i>mg. per 100 cc.</i>	<i>L:C</i>		<i>mg. per 100 cc.</i>	<i>L:C</i>
Dec. 31	52.5	0.525	Dec. 18		
Jan. 4	325	5.85	" 19	57.2	0.791
" 5	500	4.00	" 20	106	1.69
" 6	245	2.45	" 21	1390	22.3
" 7	169	2.58	" 22	400	6.66
" 8	160	2.32	" 23	335	4.85
" 9	260	1.82			
" 10	75	2.07			
" 11	250	2.50			
" 12	146	2.90			
Jan. 25	95	1.16	Jan. 27	68.5	1.02
" 26	93	1.19	" 28	34.5	0.940
" 27	112	1.12	" 29	91.0	1.00
" 28	55.2	1.24	" 30	40.5	1.05
" 29	41.7	1.21	" 31	53.5	0.900
" 30	160	1.20	Feb. 1	73.0	0.981
Mar. 9	142	0.855	Feb. 29	8.9	0.902

\* L:C = lactose to creatinine ratio.

the L:C ratios were at a constant and lower level. When the urines were tested again at a still later date during lactation, it was found that the L:C ratios were approaching an even lower level, though still somewhat higher than the RR:C values for normal women and men.

It would seem, therefore, that during the first few weeks after the mammary glands come into full activity, some adjustment must be made by the individual. The sudden excretion of lactose by many women on the 2nd to the 4th day might be interpreted to indicate that when milk secretion first starts in, this adjustment has not been made and consequently large amounts of lactose are poured into the circulation and excreted. As the adjustment sets in, the lactose excretion gradually assumes a constant and lower level.

From the data collected, therefore, it would seem safe to assume

that, during the course of lactation, some adjustment is going on which results in a constant decrease in the amount of lactose being excreted, and that the value for the L:C ratios will depend upon the efficiency of this adjustment rather than upon the degree of activity of the mammary glands. This interpretation rationalizes the whole picture. It is not worth while to make any hypothesis as to the nature of this adjustment on the basis of the present data.

*Daily Lactose Excretion of Pregnant Women up to Day of Delivery.*

On nine women who were in the last weeks of pregnancy, the daily level of the residual reduction of the urine was followed up to the day of delivery. All of these women had normal deliveries. Representative results are given in Table III.

In this particular phase of the investigation we are especially at a loss for any interpretation of the data obtained. In the first

TABLE III.  
*Daily Urine Analyses on Pregnant Women.*

Name.....	Mrs. R. P.			Mrs. Breslin.		
Remarks.....	Normal delivery May 5. Definitely deficient in milk.			Toxemia. Normal delivery Feb. 22. Excess of milk.		
Days before delivery.	Date.	Residual reduction of urine.		Date.	Residual reduction of urine.	
		mg. per 100 cc.	RR:C*		mg. per 100 cc.	RR:C
56	Mar. 11	50	0.807	Feb. 10	320	2.01
12				" 11	133	2.06
11				" 12	142	1.20
10	Apr. 25	40.6	0.711	" 13		
9	" 26	66.6	0.730	" 14	364	1.82
8	" 27	94.5	0.705	" 15		
7	" 28	59	0.940	" 16	262	1.83
6	" 29	53.4	0.693	" 17		
5	" 30	35.2	0.422	" 18	364	1.82
4	May 1	52.8	0.550	" 19	80	1.60
3	" 2	41.8	0.710	" 20	663	2.65
2	" 3	63	0.760	" 21	842	3.79
1	" 4	63	0.852	" 22	748	4.60
Delivery.	" 5	108	1.20			
1st day after delivery.	May 6	35	0.442	Feb. 23	38.6	1.28

TABLE III—*Concluded.*

Name.....	Mrs. P. B.			Bernice B.		
Remarks.....	Normal delivery Dec. 2. Plenty of milk.			Still-born baby Mar. 14.		
Days before delivery.	Date.	Residual reduction of urine.		Date.	Residual reduction of urine.	
		<i>mg. per 100 cc.</i>	<i>RR:C</i>		<i>mg. per 100 cc.</i>	<i>RR:C</i>
12	Nov. 20	81.7	0.640			
11	" 21	144	0.789			
10	" 22	141	0.938			
9	" 23	98.2	0.859			
8	" 24	70	0.862			
7	" 25	46.4	0.928			
6	" 26	96.8	1.11	Mar. 8	53	0.640
5	" 27	190	1.03	" 9	183	0.870
4	" 28	81.5	0.903	" 10	40	1.05
3	" 29	196	1.08	" 11	180	1.48
2	" 30	175	1.07	" 12	133	1.52
1	Dec. 1	164	1.29			
Delivery.	" 2	24.5	1.44	" 14	163	2.44
1st day after delivery.	Dec. 3	47.2	0.842	Mar. 15	119	0.650

\* RR:C = residual reduction to creatinine ratio.

place, with these urines, most of the values for residual reduction are so low that we do not feel justified in labelling them lactose. On the other hand the RR:C ratios are at a level definitely higher than the normal, and the natural assumption would be that these high values are due to a more or less constant excretion of small amounts of lactose. The strongest support that we have in our own findings for this assumption is the data on the first late pregnancy case studied. This patient, Mrs. Breslin (Table III), a toxemia patient, came to the hospital 3 weeks before delivery, showing a "positive sugar" test in the urine. Analysis of her urines showed that the sugar excreted was non-fermentable, and in large enough amounts (200 to 800 mg. per cent) to justify calling it lactose. It seems logical to consider this an exaggerated case of a common phenomenon, especially since many of the other cases showed at times a residual reduction of the urine amounting to more than 160 mg. per cent. It is difficult to imagine any other non-fermentable reducing substance (that would show up by the



method used) which would be excreted at this time. Also, earlier workers have been able to identify lactose in the urines of pregnant women. Porcher (10), in his observations on women, noted, in several cases, traces of lactose (identified by osazone formation) in the urines of women during the last few days before delivery. His method depended upon the presence of relatively large amounts of lactose. His earlier (8) observations on cows, and Campus' (19) more recent experiments upon these animals may be offered as further proof that, in mammals, lactose is often excreted during the last stages of pregnancy. Both of these workers have found large amounts of lactose in the urines of cows just before labor, and appreciable amounts as much as 16 days before, which is the earliest that analyses were run. The excretion of milk sugar, they report, increases steadily up to the day of calving, at which time it reaches its maximum. Campus has further added that the data on lactose in the urine prior to delivery do not justify a prediction of the subsequent milk production. These data on cows find an extraordinarily close parallel in our findings on women, although in women the lactose excretion is at a much lower level.

On the basis of the foregoing considerations, therefore, it would seem safe to assume that the high level of the RR:C ratios observed in the urines of pregnant women is due to a more or less constant excretion of small amounts of lactose.

. With several of these cases the urine was followed for a month or more before delivery, and the RR:C ratios over this period showed a certain amount of fluctuation, but were all decidedly higher than normal values. The most significant change in RR:C level comes during the last days before delivery. In all of the cases studied, the lactose excretion began to rise on the 3rd to 6th day before delivery and reached its maximum on the day when labor set in. On the day after delivery there is a sharp drop to a level which in most cases is lower than that shown at any other time, either before or after delivery. In all but two of the cases studied the lactose excretion on the day of delivery is higher than at any previous times. In these nine cases, the urine analysis was continued for several days after delivery, and it was observed that a slight rise in lactose excretion just before delivery does not necessarily mean that the after delivery lactose excretion will be correspondingly low. Also a very marked rise in the RR:C

ratios during the last days before delivery may be followed by a comparatively low level of lactose excretion during the first 2 weeks of lactation. This fact, that there is no parallel between the lactose excretion before and the lactose excretion after delivery, would seem to show that these two phenomena are not closely connected, and an explanation that would clear up one would probably not apply to the other.

It was also demonstrated by the data collected that findings on lactose output in the urine prior to delivery did not justify a prediction of the subsequent milk production. As would be expected, when the mammary glands are definitely deficient (as in the case of Mrs. R. P., Tables I and III), the RR:C ratios are low both before and after delivery. Also (as in the case of Mrs. Breslin, Tables I and III) a large amount of milk during lactation may be associated with a marked excretion of lactose both before and after delivery. But the fact that the two do not necessarily run parallel was demonstrated by the other cases studied. This would seem to indicate that the phenomenon is not associated with the degree of activity of the mammary glands.

We are at a loss for any interpretation of these prior to delivery data. The after delivery fluctuations in lactose excretion seem to be due to the inability of the system to adjust itself immediately to the large amounts of lactose secreted when the mammary glands come into full activity. On this same basis, the rise in lactose excretion just before delivery might be interpreted to signify an increased activity of the mammary glands at this time. From this point of view, the fall in lactose excretion after delivery would be taken to indicate that the mammary glands cease all activity at this time and then become active again a day or two later. To us, this does not seem an adequate explanation.

Another possibility that suggests itself is that there might be a decrease in lactose tolerance just before delivery. In our later data, however, on tolerance for ingested lactose during pregnancy, we were not able to obtain evidence that the tolerance for lactose does decrease at this time when the RR:C ratio rises. The reason for this rise in lactose excretion just before delivery is therefore entirely obscure. Whether it represents an increase in lactose secretion or a decrease in the ability of the system to take

care of lactose without the help of excretion, it is an entirely unexplained phenomenon.

It is still a mystery to obstetricians what the mechanism is that brings on labor and delivery. Some say that when the fetus reaches a certain size, labor is stimulated. Others have ascribed it to an upset in the endocrine equilibrium at this time. In view of the fact that the whole question is still so much in the dark, the foregoing findings would seem to be of especial interest, while any interpretation of them is impossible.

#### *Lactose Tolerance Tests.*

Earlier workers who have experimented upon the urinary excretion following the injection or ingestion of lactose arrived at the conclusion that when lactose or galactose reached the circulation as such they were completely unassimilable. More recent work, especially that of Folin and Berglund (20), has laid this theory open to question. The fact that the tolerance for galactose is increased by the simultaneous ingestion of glucose would seem to show that, in the normal ingestion of lactose, when glucose and galactose supposedly enter the circulation in equivalent amounts, a certain amount of utilization results. Moreover, the enzymatic splitting of lactose into glucose and galactose is limited in the adult intestine, and the ingestion of this carbohydrate is likely to result in the introduction of lactose as such into the circulation. Folin and Berglund (20) have shown that in the male adult the ingestion of 30 gm. of lactose results in the excretion of some unsplit lactose. It is possible to picture that the ingestion of smaller amounts of lactose might result in the introduction of some of this carbohydrate into the circulation as such, and to attribute the failure of it to appear in the urine to an ability of the organism to utilize it in limited amounts.

This is a purely hypothetical suggestion, but if it could be shown that individuals varied in their ability to assimilate lactose, then one would be justified in assuming that the ability to utilize this sugar when introduced into the circulation was probably never entirely lacking. In the course of our work on lactose tolerance tests, it has been observed that, among males, different individuals vary somewhat in their urinary response to ingested lactose, and that, among females, not only is this true, but the same individual

may show a variance in her tolerance toward this carbohydrate at different times. This is offered as support of the suggestion made by Folin and Berglund (20) that the ability to utilize lactose when introduced into the circulation is probably never so entirely lacking as the earlier workers supposed.

However, the main object of these lactose tolerance experiments was not to attempt to prove that lactose was assimilable, but rather to discover what we could about the response of women to the ingestion of lactose, in the hope that it might give some clue to their metabolism of this carbohydrate. It was at first thought that the phenomena in lactose excretion connected with pregnancy and lactation might be attributed to changes in the ability to utilize lactose. Our results on lactose tolerance tests have not shown this to be the case, but they have brought to light certain changes in lactose metabolism which accompany sexual activity in women.

The work of Folin and Berglund (20), in 1922, was the first that was done on lactose tolerance with the use of modern quantitative methods. Practically all of the work which has been done on this subject since that time has been carried out in the attempt to discover the nature of the inter-relationship of glucose and galactose in lactose metabolism. The results of these workers, therefore, have no particular bearing upon our own investigation, especially since all of their experiments have been upon male subjects.

Corley (21), 1927, 1928, in his articles on lactose metabolism, decided to work only on male rabbits, for, he states, "the results with female rabbits have not been consistent." This observation is quite suggestive in the light of our own findings on women. Corley himself suggests that the inconsistent results with female rabbits might be associated in some way with differences in galactose utilization at different stages of the sexual cycle or sexual development. The findings which are about to be described show that Corley's hypothesis was undoubtedly correct.

The only recent work on tolerance which has been along the same lines as our own investigation is that of Rowe which has appeared in the literature from time to time during the last 4 years under the general heading of "The Metabolism of Galactose" (22-25). Rowe's work has been entirely on galactose tolerance; that is, the urinary excretion following the ingestion of galactose. He has been particularly interested in the metabolic problems of women, and therefore his work should be of especial interest to us. Unfortunately, however, Rowe's analytical methods are not adequate enough to justify all of the conclusions which he draws. His tolerance test consists of qualitatively testing the urine for "sugar" once before and twice during the 4 hours after the ingestion of 30 or 40 gm. of

galactose. If the urine is qualitatively positive, according to Benedict's test, a quantitative titration is run, but no attempt is made to ascertain the volume or concentration of the urine. Our own work on method has shown that not even a quantitative determination is sufficient in itself to give any indication of the actual sugar excretion. Some method of keeping track of the concentration is necessary. Most workers have done this, in tolerance tests, by collecting the hourly urine specimens and reporting the sugar excretion in mg. per hour. Rowe's omission in using any such method makes his results less valuable than they might otherwise have been. Any tremendous changes in galactose tolerance would probably show up in Rowe's test, and therefore as an aid to the clinical diagnosis of endocrine disorders it may be useful. But the test is not sufficiently instructive to be used as a basis for the conclusions that Rowe draws concerning the metabolism of galactose in normal men and women.

In this investigation the method used in testing the tolerance for lactose has been the following. Whenever possible the subject has gone without breakfast, but we have found that by the 2nd hour after breakfast any glycuressis has disappeared and, therefore, among our volunteers, we have not insisted upon this point. Hourly urine specimens were collected for 2 hours before and for 2 or 3 hours after taking the weighed amount of lactose. The subjects fasted during the course of the experiment. The lactose, dissolved in 100 to 150 cc. of water, was taken immediately after the second urine specimen was passed. In all tests, pure bacto-lactose was used, a standardized product put out by the Digestive Ferments Company.

In the first tests made, both the excretion of lactose in mg. per hour and the hourly RR:C ratios were determined, and in all cases where we were sure that the total hourly specimens were carefully collected these two forms of expressing the results checked. But in many of the subjects we could not be sure that the exact hour of collecting the specimen would be kept track of, or that the total specimen was always saved. It was therefore decided to keep all results in terms of RR:C ratios. It is perfectly evident, from the data collected, that the rise in RR:C level is a response to the ingested lactose. We have not attempted to differentiate between lactose and galactose excretion, since this phase of the problem does not particularly concern this investigation. Since most of the doses of lactose were less than 30 gm., however, we assume, on the basis of Folin and Berglund's (20) results, that the rise in RR:C level represents galactose

excretion. Our particular concern is the utilization of lactose, and if the ingestion of a given amount does not result in urinary excretion of either lactose or galactose, it seems safe to assume that this sugar has been assimilated.

*Lactose Tolerance of Normal Male Adults.*

For the purposes of comparison, tests were first run on nine normal men. Folin and Berglund (20) report 10 gm. of lactose as the tolerance dose for the normal male adult. Our own data check with this finding. Table IV represents a typical case.

TABLE IV.

*Urinary Response of a Normal Male to Ingestion of Lactose.*

Subject P.L.

Time.	Remarks.	Experiment 1. 20 gm. lactose.		Experiment 2. 10 gm. lactose.		Experiment 3. 5 gm. lactose.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		mg. per 100 cc.	RR:C*	mg. per 100 cc.	RR:C	mg. per 100 cc.	RR:C
9.00 a.m.	No breakfast. Fasting.	80	0.391	56.8	0.410	80	0.520
10.00 "	"						
11.00 "	Took lactose.	40	0.360	76	0.360	54	0.415
12.00 "	Fasting.	48.5	1.01	40	0.600	57.2	0.388
1.00 p.m.	"	8	0.436	12.5	0.448	78.5	0.415
		52.2	0.410	53.5	0.322	84	0.420

\* RR:C = residual reduction to creatinine ratio.

The results of Folin and Berglund are in terms of total sugar excretion in mg. per hour. In all of our tolerance tests we have analyzed for total sugar as well as residual reduction, and although the two do not always run exactly parallel, in general the same results would have been acquired for tolerance dose whether the urine analyses were reported in terms of TS:C or RR:C ratios. However, the urinary response to the ingestion of lactose is usually more decidedly evident when the results are expressed in terms of residual reduction rather than total sugar.

The fact that the residual reduction in mg. per 100 cc. gives absolutely no indication of the actual level of the excretion is more

than evident. We have included these figures in the tables, however, since, in connection with the R.R.:C ratios, they give an indication of the concentration of the urine specimens, and show whether or not diuresis followed the ingestion of lactose. The fact that, in the case of P. L. (Table IV), diuresis occurred in the two experiments where the urinary response to the ingestion of lactose was evident, is not to be taken as significant, for the results on other individuals show that this is by no means the rule.

Although 10 gm. may be considered the tolerance dose for the normal male, we have found that all men do not respond to the ingestion of this amount of lactose with the same degree of rise in the R.R.:C level of the urine. The response of one of the subjects was barely determinable, the rise of the R.R.:C level being only from 0.270 to 0.298. Another showed a rise in R.R.:C level from 0.335 to 0.695 after the ingestion of 10 gm. of lactose. The others ranged between these two extremes. In practically every case the R.R.:C level was back to normal by the end of the 2nd hour after ingestion of the lactose.

These tests on men were carried out for the purposes of comparison and will be referred to again in connection with the results of like tests on women.

*Lactose Tolerance of Normal Women at Different Stages of the Sexual Cycle.*

It was not at first expected that any difference would be found between the response of normal men and that of normal women to the ingestion of lactose. It was therefore a surprise to discover, in the case of the first test tried on a normal woman, O. W., that the individual showed no urinary response to the ingestion of 20 gm. of lactose. No men have been observed who did not respond to 10 gm. However, when it was attempted at a later date to repeat this observation on O. W., it was discovered that the individual not only responded to 20 gm., but showed as great a response to 10 gm. as did any of the normal men. It naturally occurred to us at this point that the tolerance for lactose in women might be influenced by the sexual cycle, and our results on the seven women studied showed that this is the case. These seven women varied in age from 23 to 33 years, and all of them are unmarried.

TABLE V.

*Urinary Response of Normal Women to Ingestion of Lactose.*

Subject O. W.; response to ingestion of 20 gm. lactose.

Time.	Remarks.	Intermenstrual period. 11th day of sexual cycle.		2nd day before menstruation. 28th day of sexual cycle.		2nd day of menstruation.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		<i>mg. per 100 cc.</i>	<i>RR:C*</i>	<i>mg. per 100 cc.</i>	<i>RR:C</i>	<i>mg. per 100 cc.</i>	<i>RR:C</i>
7.30-10.30 a.m.	Breakfast at 8.00 a.m. Fasting.	70	0.557	15.4	0.723	38.2	0.395
11.30 a.m.	Took lactose.	33.5	0.520	16.7	0.556	36.5	0.393
12.30 p.m.	Fasting.	12.3	0.950	53.3	0.374	10.2	0.432
1.30 "	"	33.7	0.500			34.2	0.425
2.30 "	"	13.4	0.500	28.2	0.425	22.6	0.405

Subject J. D.; response to ingestion of 10 gm. lactose.

Time.	Remarks.	Intermenstrual period. 8th day of sexual cycle.		2nd day before menstruation. 26th day of sexual cycle.		1st day of menstruation.	
8.30-9.30 a.m.	Breakfast at 7.30 a.m.			25.8	0.602		
10.30 a.m.	Fasting.	80	0.480			32.5	0.440
11.30 "	Took lactose.	69.5	0.450	79	0.685	37.5	0.400
12.30 p.m.	Fasting.	55	0.550	84	0.650	36.2	0.415
1.30 "	"	61.5	0.382	94	0.640	61.2	0.350

Subject M. E.; response to ingestion of 10 gm. lactose.

Time.	Remarks.	Intermenstrual period. 11th day of sexual cycle.		2nd day before menstruation. 26th day of sexual cycle.		2nd day of menstruation.	
8.00-10.00 a.m.	Breakfast at 8.00 a.m. Fasting.	51.5	0.325	153	0.602	45.5	0.455
11.00 a.m.	Took lactose.	59	0.322	80	0.677	33	0.420
12.00 noon.	Fasting.	20	0.445	32.7	0.570	22.2	0.400
1.00 p.m.	"	13.5	0.292	12.1	0.660	18.2	0.406

\* RR:C = residual reduction to creatinine ratio.



At first no special plan was followed as to the time of making the tests, since we had no idea what to expect. But it was soon evident that it was just before and during menstruation that the effect on lactose tolerance was marked. Ovulation does not seem to be concerned with this phenomenon. The course followed therefore was to find out what the tolerance was in the intermenstrual period, and then to test out the response to this amount of lactose once just before and once just after the beginning of menstruation. Representative results are given in Table V.

TABLE VI.

*Urinary Response to the Ingestion of Lactose of a Woman Who Apparently Is Not Normal.*

Subject E.P.; response to ingestion of 10 gm. of lactose.

Time.	Remarks.	Intermenstrual period. 12th day of sexual cycle.		4th day before menstruation. 26th day of sexual cycle.		3rd day of menstruation.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		mg. per 100 cc.	RR:C*	mg. per 100 cc.	RR:C	mg. per 100 cc.	RR:C
7.30-9.30 a.m.	Breakfast at 7.30 a.m.	88	0.486			66	0.365
10.30 a.m.	Fasting.	92	0.498	63	0.380	75	0.355
11.30 "	Took lactose.	55.3	0.499	59.9	0.387	58	0.385
12.30 p.m.	Fasting.	407	3.00	286	2.46	305	2.15
1.30 "	"	139	0.940	128	0.605	96.2	0.582

\* RR:C = residual reduction to creatinine ratio.

Only two subjects were found who did not show, in the intermenstrual period, a response to 10 gm. of ingested lactose, the rest of the normal women showing, at this time, a urinary response which was entirely comparable to the response of normal men. The effect of the sexual cycle upon the lactose tolerance was evident in every case studied. In most cases the effect is most marked just before menstruation, and, in all but one of the cases studied, the tolerance has been found to be increased at this time to such an extent that there is no response to the ingestion of 10 gm. of lactose, and in some cases no response to the ingestion of

20 gm. During menstruation also, it has been found that the tolerance is greater than during the intermenstrual period.

In the course of our work upon the lactose tolerance of normal women we have happened upon one individual whose tolerance is decidedly abnormal. Table VI gives the results of three of the tests made upon her.

The data show that even this abnormal subject is affected in her lactose tolerance during menstruation, the rise in RR:C ratio being less at this time than in the intermenstrual period. As far as E.P. knows, she has no physical abnormalities. She shows no signs of glycosuria, nor is her glycuressis after meals any more marked than that of the normal individual,—except, of course, when the meal contains any amount of milk. The only illness in her history is a very serious nervous breakdown 3 years ago, from which she has entirely recovered. In the light of Rowe's work on galactose tolerance (24), it is entirely possible that E.P.'s abnormally low tolerance for lactose is due to some endocrine disorder. According to Rowe, increased function of the posterior lobe of the pituitary, ovarian failure, or adrenal failure may cause a lowering of the tolerance for galactose.

From the data on E.P., it is seen that her abnormal response to lactose ingestion would be evident even if only a qualitative test of the reducing power of the urine were used, since anything over 150 mg. per 100 cc. gives a positive test with Benedict's reagent, the one used by Rowe in his experiments. It is equally evident, in looking over the residual reduction in mg. per 100 cc. in Table V, that Rowe's method of making the tolerance test would be inadequate as an indication of the changes in tolerance of normal women. Rowe reports that the tolerance dose of normal women is in general 10 gm. higher than that of normal men, and that during menstruation the tendency is for the tolerance to *decrease*. He places the normal tolerance dose at 30 gm. for men and 40 gm. for women. It is not surprising that Rowe's results do not agree with our own when one considers the difference in methods. The fact that Rowe feeds galactose while we are feeding lactose would probably not explain the differences in results, since it has been found (Folin and Berglund) that the tolerance for galactose is even lower than that for lactose. In lactose feeding it is probable that the principal sugars that reach the circulation are galactose and glu-

cose. It is therefore safe to assume that any changes in tolerance observed in women during sexual activity would be in the same direction whichever sugar were fed.

We do not attempt to offer any explanation why the lactose tolerance of normal women should increase at the time of menstruation. Rowe interprets his findings of higher tolerance for galactose in women than in men as indicating that the mammary glands are able to absorb this sugar. It might, of course, be possible that at the time of menstruation some mammary activity went on which caused the absorption of galactose. It seems, however, to have been pretty well proved that the mammary glands use only glucose in the secretion of milk (Porcher (9)), and there is therefore no real reason to suppose that they would absorb galactose at any time. Rowe's own findings of the profound effect of endocrine abnormalities upon galactose tolerance suggest a more likely source of explanation, especially since the endocrine glands are so clearly associated with sexual phenomena. However, we merely present the facts which our observations have made clear; namely, that in most normal women the lactose tolerance is the same as that of normal men, except just before and during menstruation, at which time it is increased.

#### *Lactose Tolerance of Pregnant Women.*

The finding of an increased tolerance for lactose in women at the time of menstruation led us to expect that pregnancy would surely have some effect upon the urinary response to the ingestion of this carbohydrate. It was not surprising, therefore, to find that a marked increase in the tolerance for lactose is coincident with pregnancy. Among the seventeen pregnancy cases followed there was only one that showed any urinary response to the ingestion of less than 20 gm. of lactose. In a number of the cases the response to 20 gm. was very slight (less than the normal response to 10 gm.), and with five of the pregnant women 30 gm. were found to be the tolerance dose.

For this part of the investigation we were extremely fortunate in having at our disposal, through the kindness of Dr. Foster Kellogg, the Florence Crittenton Home Hospital, where normal pregnancy cases are accommodated for as many as 5 months before delivery and 2 months after. All of these subjects went without breakfast on the days that the test

was tried, and, thanks to the very intelligent cooperation of the nurse in charge, we could always be certain that the tests would be carefully carried out.

TABLE VII.  
*Urinary Response of Pregnant Women to Ingestion of Lactose.*

Mrs. Noring. Normal delivery Mar. 1. Milk sufficient.							
Time.	Remarks.	10 gm. lactose Feb. 21.		20 gm. lactose Feb. 23.		30 gm. lactose Feb. 24.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		mg. per 100 cc.	RR:C*	mg. per 100 cc.	RR:C	mg. per 100 cc.	RR:C
6.00 a.m.	Fasting.	48.5	0.760	53.4	0.960	49	0.620
7.00 "	Took lactose.	15.8	0.789	76.1	0.886	45	0.605
8.00 "	Fasting.	51.5	0.762	73	0.880	49	0.830
9.00 "	"	44	0.750	76.1	0.882	48.5	0.720
10.00 "	"	26.8	0.750	66.2	0.860	48.5	0.655
Lillian. Normal delivery May 11. Milk sufficient.							
Time.	Remarks.	20 gm. lactose Mar. 23.		10 gm. lactose Mar. 28.		20 gm. lactose May 9.	
6.45-7.45 a.m.	Fasting.	30	0.605	33.3	0.880	44	0.628
8.45 a.m.	Took lactose.	34.5	0.590	36.5	0.840	31.7	0.606
9.45 "	Fasting.	91	0.800	26.6	0.820	76.2	0.671
10.45 "	"	108	0.700	114	0.850	71.1	0.690
11.45 "	"	100	0.580	118	0.855	69	0.621
Edith I. Due to be delivered on Aug. 15.							
Time.	Remarks.	20 gm. lactose Mar. 29.		10 gm. lactose Mar. 30.		20 gm. lactose May 10.	
6.45-7.45 a.m.	Fasting.	24	0.630	12.6	0.765	113	0.705
8.45 a.m.	Took lactose.	20	0.538	89	0.750	46.2	0.694
9.45 "	Fasting.	40	0.535	114	0.745	68.4	0.718
10.45 "	"	69	0.670	160	0.740	95.2	0.876
11.45 "	"	50.8	0.735	131	0.755	114	0.886

\* RR:C = residual reduction to creatinine ratio.

The procedure followed on pregnancy cases was first to try the test with 20 gm. of lactose; if there was no urinary response to repeat the test with 30 gm. If, on the other hand, there was a

response to 20 gm., 10 gm. were tried in order to make sure that 20 gm. were the tolerance dose. In the cases of early pregnancy, the test was repeated at a date nearer to delivery to see if the tolerance changed as pregnancy advanced. Representative results are given in Table VII.

The most noticeable difference in lactose excretion between normal and pregnant women, aside from the increased tolerance of the latter, is in the delayed rise in the curve for the residual reduction of hourly urine specimens after lactose ingestion. In normal women, as in normal men, the maximum rise in RR:C level comes during the 1st hour after the ingestion of lactose, and in almost every case, with normal men, and with women whether menstruating or not, the RR:C level is back to normal by the end of the 2nd hour after the lactose was taken. This is quite evidently not true with pregnant women. In many of the cases studied the maximum response is not until the 2nd or 3rd hour after ingestion, and the return to normal is likewise slower than in the normal individual.

It has been quite evident from the results on the cases tested at the end of pregnancy, which we have been able to observe after delivery, that the amount of increase in tolerance during pregnancy is in no way related to the amount of milk that the women have for their babies. It almost seems, therefore, that here again the explanation for the phenomena observed would not be found to have anything to do with the degree of activity of the mammary glands.

As far as we could discover, there is no relation between the tolerance for lactose and the daily RR:C ratios during pregnancy. In several cases the tolerance test was made a day or two before delivery when the RR:C level had begun to rise, but in general it was found that at this time the tolerance for lactose was as high or higher than it had been earlier, when the RR:C ratios were lower. The increase in lactose excretion which has been observed to occur just before delivery cannot, therefore, be attributed to a decrease in lactose tolerance.

In some of the cases followed it appeared that the lactose tolerance increased as pregnancy advanced, and that the tendency was for the urinary response after the ingestion of lactose to be slower in manifesting itself as the time for delivery approached.

However, it was evident from our data that no generalization can be made upon this point.

As in the case of the effect of menstruation, here again our results do not agree with those of Rowe. Rowe finds that the tolerance of pregnant women for galactose, especially in the later stages, is markedly lowered. An examination of our own data on this part of the investigation made it quite evident how easy it would be to draw incorrect conclusions concerning the tolerance of pregnant women if one used a method of testing the urine which did not take into account the concentration of the specimen. In the first place the reducing power of the urines of pregnant women is in general higher than that of normal women as we have already shown, so that a smaller rise in reducing power would push it above the value where it would give a qualitative test for sugar. In the second place (as is seen from the cases given in Table VII), with pregnant women, the urines after the lactose is taken are in many cases more concentrated than before. This would make it possible to get a qualitatively positive test when actually the rate of sugar excretion had not changed. The tendency toward dehydration after the ingestion of lactose among pregnant women is, in itself, worthy of note. Among normal women and men, diuresis is more likely to occur.

Rowe ascribed the increased tolerance of normal women, which he observed, to a capacity for storage of galactose in the mammary glands. His interpretation of his finding of a "lowered tolerance during pregnancy" is that the mammary glands have started their synthetic activity and have gradually become saturated so that their storage capacity is practically eliminated. We shall make no attempt to interpret our findings on the changes in lactose tolerance coincident with pregnancy. However, we are inclined not to ascribe these changes to mammary activity, especially in the light of the results obtained from tolerance tests on lactating women.

#### *Lactose Tolerance of Lactating Women.*

The uniformity of the results in this part of the investigation was rather surprising, and left no question as to the urinary response of lactating women to the ingestion of lactose. In all of the ten cases experimented upon, 10 gm. of lactose were found to be

the tolerance dose. Also the type of curve for the hourly RR:C ratios was the same as that found in normal women and men, the maximum response coming the 1st hour after ingestion, and the return to normal being complete, in most cases, by the end of the 2nd hour after the lactose was taken. Representative results are given in Table VIII.

With six of the cases the tolerance dose before delivery had been determined. Four of these had shown, during pregnancy, a tolerance dose of 20 gm., one of 30, and the other of 10 gm. of lactose. The tolerance of lactating women seems to be in no way

TABLE VIII.  
*Urinary Response of Lactating Women to Ingestion of Lactose.*

Time.	Remarks.	Mrs. P. B. Normal delivery Dec. 2. Enough milk most of time.		Bernadetti. Normal delivery Mar. 20. More than enough milk.		Rebecca B. Normal delivery Apr. 13. Milk often insufficient.	
		10 gm. lactose Feb. 14.		10 gm. lactose Apr. 10.		10 gm. lactose May 16.	
		Residual reduction of urine.		Residual reduction of urine.		Residual reduction of urine.	
		<i>mg. per 100 cc.</i>	<i>RR:C*</i>	<i>mg. per 100 cc.</i>	<i>RR:C</i>	<i>mg. per 100 cc.</i>	<i>RR:C</i>
6.45-7.45 a.m.	Fasting.	89	1.08	193	1.56	28	0.460
8.45 a.m.	Took lactose.	59.3	1.01	177	1.59	40	0.410
9.45 "	Fasting.	50	1.20	145	1.72	48.2	0.590
10.45 "	"	17.7	1.12	156	1.56	52.6	0.472

\* RR:C = residual reduction to creatinine ratio.

connected with what the tolerance of the individual was before delivery, nor with the amount of milk for the baby, nor with the daily level of the RR:C ratios either before or after delivery. In all cases 10 gm. of lactose are the smallest amount which will cause a rise in the RR:C ratio of the urine.

Rowe examined ten women during the first 2 weeks after delivery and found their tolerance very low. We have not attempted to test women during this period since we considered that the high lactose excretion which is evident at this time, and especially the great fluctuations in L:C ratios, made any tolerance test in which the changes in level are so small, entirely unpracticable. For

these same reasons we are inclined to question Rowe's results on this point, and to ascribe his finding of a low tolerance to the fact that the reducing power of the urines of lactating women at this time is usually very high. Our earliest test on a lactating woman was made 3 weeks after delivery, and the latest test was  $2\frac{1}{2}$  months after delivery. We are inclined to believe that immediately after delivery the lactose tolerance of women returns to the normal 10 gm. level shown by normal men, and by most normal women in the intermenstrual period.

It would seem, therefore, that after delivery, during the months of lactation, whatever influences cause the increase in tolerance for lactose at the time of menstruation and during pregnancy have ceased to exert themselves. The mammary glands, during lactation, are at the height of their activity, and the fact that the tolerance is unchanged would appear to furnish rather strong support of the previous hypothesis, that the phenomena observed in the course of this investigation probably cannot be considered as due to the degree of activity of the mammary glands.

#### DISCUSSION AND RÉSUMÉ.

This investigation is principally concerned with the presentation of data on lactose excretion in women. Little in the line of interpretation is possible, but the accumulation of accurate results is necessary for future work on the subject, and helps eliminate the possibility of drawing incorrect conclusions.

It has been found that during pregnancy and lactation, lactose is found in the urines of women in varying amounts. During the first few weeks after delivery lactose is often excreted in very large amounts (as high as 4 per cent) and the fluctuations in excretion from day to day are marked. There is evidence that this excretion starts in when the mammary glands first assume full activity and that it is due to a lack of some sort of adjustment which later becomes evident and causes the residual reduction to creatinine (RR:C) ratios of the urine to assume a constant and lower level. It has been found that the tolerance for ingested lactose at this time is the same as in normal individuals, and therefore the gradual decrease in lactose excretion as lactation advances cannot be considered as due to an increase in lactose tolerance. It is possible that the adjustment is in the mammary



glands themselves. It seems reasonable that in the course of lactation, the mammary glands should adjust themselves to produce exactly as much lactose as is needed for the milk and no more, so that there would be little or no escape of excess lactose into the circulation. The fact that during the first 2 or 3 weeks the adjustment is so far from complete, and that even at the end of 3 months there is still some excretion of lactose is not, however, in keeping with the usual efficiency of the animal machine.

The appearance of lactose in the urine during pregnancy cannot be ascribed to a decrease in the tolerance for this sugar for tolerance tests show that in pregnancy the tolerance for lactose is markedly increased. It is possible, however, that owing to a certain amount of mammary activity at this time, lactose is present, and, although the tolerance is high, showing that some assimilation is possible, it is not so high but that a little lactose escapes into the blood stream and appears in the urine.

A striking increase in lactose excretion just before delivery has been observed. It has been shown that this phenomenon is not related to the degree of activity of the mammary glands, nor to the lactose excretion after delivery. Also, the experimental evidence seems to show that the lactose tolerance does not decrease at this time. Whatever the explanation of this increased excretion may be, it seems quite possible that it is caused by the same metabolic change which brings on labor and delivery. This latter phenomenon is at present entirely unexplained, and for this reason the foregoing observation is particularly suggestive.

The results on lactose tolerance tests have brought to light many peculiarities in the lactose metabolism of women, although they do not appear to furnish any explanation of the observations on the daily level of lactose excretion during pregnancy and lactation. These tolerance tests do, however, provide further evidence of the fact that there is a decided upset in the metabolism of lactose associated with female sex activity.

The natural assumption would be that all the phenomena observed must be associated with the mammary gland, since this organ is concerned with the secretion of lactose. It was to the mammary glands that Rowe turned in the interpretation of his findings. From the data which we have been able to gather, however, we are inclined to doubt whether it is this gland which is

involved. None of the phenomena observed seem to be related in any way to the degree of activity of the mammary glands. Rowe (24) has found that endocrine abnormalities have profound effects upon the tolerance for galactose. Considering the fact that the endocrine glands are so closely associated with sexual activity, it seems entirely possible that the interpretation of all of the data that have been collected in the course of this investigation may lie within the field of endocrinology.

The observations that have been made, therefore, upon the urinary excretion of lactose in normal, pregnant, and lactating women demonstrate that certain peculiarities in lactose metabolism are associated with the sexual activities of women. In conclusion, a brief outline is presented of the experimental data which have been collected.

1. During the last stages of pregnancy there is a more or less constant excretion of small amounts of lactose.

2. During the last few days before delivery there is a sudden very marked rise in lactose excretion, which reaches its height on the day of delivery.

3. After delivery, the lactose excretion immediately drops to a low level where it remains for from 2 to 5 days. There is then a sudden and often tremendous excretion of lactose and, during the first few weeks of lactation, the fluctuations in the excretion of lactose are very marked.

4. By the end of the 1st month after delivery the lactose excretion has assumed a constant and lower level, and this level slowly approaches the normal values for residual reduction of the urine as lactation progresses.

5. The tolerance for lactose of most normal women is, in the intermenstrual period, the same as that of normal men, the tolerance dose being 10 gm.

6. Menstruation causes an increase in the tolerance of women for lactose, so that at this time many women show no urinary response to the ingestion of 20 gm. of lactose.

7. During pregnancy the tolerance for lactose is increased, being in some women 3 times as high as in other individuals. Also the urinary response to the ingestion of lactose is slower in pregnant women, and the return to normal is correspondingly slow.

8. During lactation the tolerance for lactose is apparently the

same in every way as it is in normal men and in most women in the intermenstrual period.

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## SOME FACTORS AFFECTING THE ADSORPTION OF QUININE, OXALATE, AND GLUCOSE BY FULLERS' EARTH AND NORIT.\*

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The purpose of this investigation was to make a study of some of the factors affecting the adsorption of electropositive, electro-negative, and non-electrolytic substances of known composition, by different adsorbents. Such a study should furnish a basis for a more efficient utilization of these adsorbents in the fractionation of the vitamin B complex. Since fullers' earth and norit had been used in this laboratory and elsewhere (1-5) for the adsorption of at least two factors of the vitamin B complex, the studies have been restricted to these adsorbents.

The available literature contains numerous references concerning the adsorption of various substances by these adsorbents, and the results have been published in some excellent summaries. Mention will be made here only of those references which relate directly to this investigation. The fact that fullers' earth carries an unusually large proportion of colloidal material of high adsorptive capacity was pointed out by Parsons (6). The ability of fullers' earth to adsorb alkaloids was probably first noted by Lloyd (7) in 1910. This investigator observed that different earths had different affinities for a given alkaloid. He attempted to isolate the active constituent from the earth, but such an attempt was unsuccessful. He found, however, that the activity could be destroyed by heating the earth to a red heat, and therefore, concluded that the alkaloidal affinity of the adsorbent depended upon its physical condition, which in turn rested upon and was closely connected with the hydrated condition of the material. Increase in hydration resulted in an increase in this affinity. Gordin and Kaplan (8) found fullers' earth to be more specific for alkaloids than other adsorbents. These investigators found that both acids and alkalies were

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adsorbed by fullers' earth and charcoal, but the presence of free acids did not affect the adsorption of alkaloids. Aluminum hydroxide was found ineffective as an adsorbent for alkaloids, glucosides, and bitter principles.

Haber (9) states that in crystals, the molecules are held together by the forces of valences, and in general these valences are satisfied; the molecules on the surface, however, are not completely saturated, and the residual valence on the surface represents the field of possible adsorption.

According to Freundlich (10) adsorption by a solid adsorbent proceeds parallel with surface energy. Michaelis and Rona (11), however, fail to agree with this general conception of adsorption. These investigators hold that there is no surface-active non-electrolyte which is adsorbed in the slightest degree by either kaolin or ferric hydroxide. Schulze (12) states that in the process of adsorption by many amorphous insoluble silicates, the total basic content of the adsorbent is replaced by bases from the solution. This view is substantiated by the findings of Gottwald (13), who combined solutions of sodium silicate and aluminum sulfate, and found that the resultant gelatinous precipitate was composed primarily of aluminum silicate contaminated with a small amount of sodium sulfate. Further light was thrown upon this type of adsorption by the investigations of Rona and Michaelis (14) who found that electroequivalent quantities of ions are given off and adsorbed during the process of adsorption. The investigations of Marc (15) show the rate at which this type of adsorption takes place and suggest that the phenomena are similar in this respect to many ordinary reactions.

The adsorption of substances by carbons appears to present even more complex phenomena. Bartell and Miller (16) believe that the reaction accompanying adsorption by carbons is hydrolytic in nature and that under certain conditions acids may be adsorbed, while under different conditions bases may be adsorbed. Freundlich and Losev (17) found that when a solution of new fuchsin in the form of its hydrochloric acid salt was treated with charcoal, the outside liquid became colorless but retained all the chlorine ions. Kolthoff (18) made a study of this type of adsorption using an ash-free sugar coal prepared according to the methods of Bartell and Miller (16) and found that this substance adsorbed electrolytes only slightly. What little adsorption did take place was attributed to the small quantities of impurities which failed to be removed, and which offered some chance for ionic exchange.

Tanner (19) brought out the similarity between the process of dyeing and the adsorption of coloring matter by carbons. According to this author, the relationship of the charge of the adsorbent to the charge of the substance adsorbed is possibly the most important factor in the dyeing process as well as in adsorption by carbons. Traube (20) found that many substances, for example organic acids, alcohols, and amines, greatly lowered the surface tension and therefore the surface energy of their aqueous solutions, which created a most favorable condition for adsorption, especially by carbons.

Although the general effect of acid and alkali upon the adsorption of

coloring matter from solutions by carbons has been known for some years, no systematic study of these factors was made until Brewster and Raines (21) studied the effect of H ion on the decolorization of cane juice. These investigators found that decolorization increased with increase in H ion concentration and advocated the adsorption of these substances at pH 4.0. More recently Blowski and Bon (22) reported similar effects upon raw cane sugar liquids.

Hauge and Williaman (23) have recently published the results of an extensive investigation of the effect of H ion concentration on the adsorption of electropositive, electronegative, amphoteric, and non-electrolytic substance by carbons. These investigators concluded that carbons adsorbed the greatest quantity of electronegative substances from the most acidic solutions, while alkalinity favored the adsorption of electropositive substances; amphoteric substances were adsorbed more strongly at their isoelectric points, while the adsorption of a non-electrolyte by carbons was unaffected by the H ion concentration of the solutions.

With the results from the investigations mentioned above in mind, we undertook to determine the effect of certain factors upon the adsorption of a cation, an anion, and a non-electrolyte by fullers' earth and norit. The major portion of our investigation has been restricted to fullers' earth as the adsorbent since this substance had previously been used rather extensively in this laboratory. Quinine, oxalate, and glucose were selected as representatives of the three different types of substances to be studied. The investigation has been confined chiefly to such factors as H ion concentration, heating the adsorbent previous to adsorption, fineness of particle, electrodialysis of adsorbent, and the extraction of the adsorbent with hot acid solution.

### *Adsorption of Quinine.*

#### *Experimental Procedure.*

A series of aqueous solutions was prepared by dissolving 1 gm. of quinine bisulfate in 500 ml. of distilled water, adding the theoretical quantity of  $\text{H}_2\text{SO}_4$  or  $\text{NaOH}$  to make approximately the desired pH, and then diluting to 1000 ml. with distilled water. All solutions which contained any undissolved quinine or showed any degree of turbidity, other than the fluorescence characteristic of this alkaloid in acid solution, were discarded. After the solution had attained equilibrium, the H ion concentration was determined by means of the hydrogen electrode. In this manner,

a series of ten solutions ranging in pH from 0.24 to 8.29, and containing 1 mg. of quinine bisulfate per ml., was prepared. It was not possible to cover the desired range of pH values owing to the insolubility of quinine in aqueous solutions more alkaline than about pH 8.29. There was prepared, therefore, another series of quinine solutions similar to the first except that aqueous alcohol (63.3 per cent by weight of ethyl alcohol) was used as the solvent. This series consisted of thirteen solutions ranging from pH 1.30 to pH 14.34 and containing 1 mg. of quinine bisulfate per ml.

After numerous preliminary tests, the following procedure was adopted for measuring the adsorptive capacity of the various adsorbents for quinine bisulfate: 0.2 gm. portions of the finely ground (150 mesh) fullers' earth were weighed accurately and placed in a number of dry 100 ml. Erlenmeyer flasks. Separate 50 ml. portions of the quinine solutions were added to each of the flasks by means of a pipette, care being taken to use a clean dry pipette for each portion. The flasks were shaken by hand at 5 minute intervals, for 25 minutes; each solution was then filtered through a small quantitative filter into a 50 ml. volumetric flask. About 20 ml. of each filtrate were used for pH measurement. Duplicate portions of 10 ml. of each filtrate were pipetted into small beakers, diluted with 10 ml. of distilled water, and made strongly acid with 1:1 sulfuric acid. The cold solutions were then titrated with  $\text{KMnO}_4$ . Blank determinations were made to determine the amounts of quinine adsorbed by the filter paper at the various H ion concentrations, also to ascertain the amount of reducing substances removed from the various fullers' earths by aqueous solutions of the same pH as the quinine solution. The data presented have been corrected whenever these errors became apparent.

The adsorption from the alcoholic solutions was carried out in a manner similar to that of the aqueous solutions. The presence of alcohol was found to complicate the permanganate titration; consequently it was necessary to remove the alcohol by evaporating the 10 ml. portions to dryness on a water-bath before titrating. In this case 20 ml. of water were added in place of the 10 ml. which were used with the aqueous solutions.

The method used in determining the excess quinine in the filtrate depends upon the oxidation of the vinyl group of the quinine molecule. This method, like other permanganate oxidation

methods, required some practice before proficiency could be attained. It was found that both the degree of acidity and the temperature of the quinine solution to be titrated were important factors in the determination. Titrating into neutral or alkaline solution was totally unsatisfactory, while titrating into hot or even warm acidic solutions resulted in high values, owing to the rupture of the nucleus by the more vigorous oxidation. It was found, however, that if the quinine solution was made distinctly acid and titrated at a temperature not exceeding 25°, consistent values could be obtained from day to day for quinine solutions of known concentrations. The oxidation progressed slowly at first, but soon accelerated and proceeded until the end-point was reached. The end-point remained visible for  $\frac{1}{2}$  to 2 hours, depending upon the quantity of alkaloid titrated. The permanganate solution used was standardized against known weights of quinine bisulfate and was of such concentration that 1 ml. of the solution was equivalent to 1 mg. of quinine bisulfate. All titrations were made in duplicate from a small burette with readings to 0.05 ml.

#### *Effect of H Ion Concentration.*

The effect of H ion concentration of the solution upon the adsorption of quinine may be observed from Fig. 1. The curves presented divide themselves into two distinct groups with regard to the quantity of quinine adsorbed. One group represents the efficient adsorption of the alkaloid by English fullers' earth, while the other group represents the far less efficient adsorption by norit and American fullers' earth. Curve A indicates the amount of quinine, expressed as mg. of quinine bisulfate, adsorbed from aqueous solutions by 1 gm. of English earth. This curve represents an average of values obtained from two samples of English earth, since the two samples were about equally effective adsorbents. The amount of quinine adsorbed reaches a maximum in the dilute acid solutions, and shows a marked decrease in the more acidic solutions and some indications of a decrease in the more alkaline solutions.

Curve C represents the composite values obtained from three samples of American fullers' earth. Likewise, these three samples were very similar in physical properties and adsorbed almost



identical amounts of quinine under the various conditions. The variation in the adsorptive capacity of the two types of earth for this alkaloid becomes apparent when we compare Curves A and C. Although the American earths adsorbed only about one-third as much quinine as the English earths, the general form of the two curves is very much the same. The maximum adsorption by the

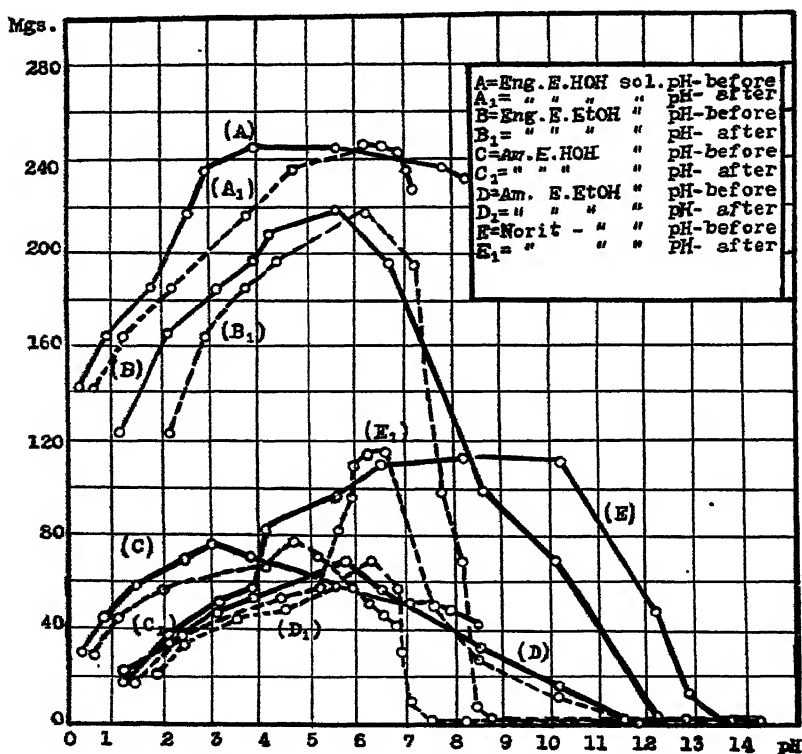


FIG. 1. Mg. of quinine bisulfate adsorbed per gm. of adsorbent at various H ion concentrations.

American earths was from slightly more acidic solutions than by the English earth. This can be explained on the basis of the difference in the composition of the two earths, as will be pointed out later.

Curve B represents the amount of quinine adsorbed by the two samples of English earth from alcoholic solutions. Although the

maximum amount of quinine adsorbed was only 88 per cent of that adsorbed from aqueous solutions of the same concentration and pH, the general trend of the two curves is very much the same. The effect of pH on adsorption from alcoholic solutions is more marked as indicated by the more abrupt rise and fall in the curve. The effect of alkalinity upon the adsorption of this substance is brought out more strikingly than was possible with the aqueous solutions.

Curve D represents the adsorption from alcoholic solutions by the American earths. These results are in full agreement with those obtained with the English earths from similar solutions. The maximum adsorption is somewhat inhibited by the alcoholic solution as was the case with the English earth, and the effect of alkalinity is equally pronounced.

Curve E shows the adsorption of quinine from alcoholic solutions by norit. This adsorbent was slightly more effective than the American earths, but removed only one-half as much quinine as was removed by an equal weight of English earth under the same condition.

The adsorption of quinine by fullers' earth appears to be a maximum just on the acid side of the isoelectric point of that earth. The slight variations in the region for maximum adsorption by different earths are due to the differences in the composition of the different earths, which result in the earths having different isoelectric points. This adsorption apparently is the result of freeing of the more active cations of the adsorbent, which in turn react with the  $\text{SO}_4$  ions. Equilibrium is established when the less active anions of the adsorbent combine with the quinine ions to form an insoluble compound or complex.

#### *Changes in H Ion Concentration.*

On making the pH determination on the various series of filtrates, it was observed that considerable pH change resulted during the adsorption period. The magnitude of these changes varied with the adsorbent used and also with the pH of the original solution. In some cases this variation was as much as 4.5 pH units. With all unpurified adsorbents, there was a decrease of acidity in the acid solutions and a decrease of alkalinity in the alkaline solutions, or a tendency to bring the solution toward the

neutral point. With such a change of acidity or alkalinity occurring during adsorption, it became apparent that the equilibrium H ion concentration (H ion concentration of the filtrate) and not the initial pH of the solution is the determining factor.

The relation between the initial and the final reaction is shown in Fig. 1; Curves A<sub>1</sub>, B<sub>1</sub>, C<sub>1</sub>, D<sub>1</sub>, and E<sub>1</sub> are plotted against the equilibrium H ion concentration. The effect of alkalinity upon the adsorption of this alkaloid becomes even more marked when the data are presented in this manner.

#### *Adsorption of Other Ions.*

To ascertain what portion of the pH change could be attributed to the removal of the quinine from the solution by adsorption, and to determine the variation in the Na and SO<sub>4</sub> ions which accompany this adsorption, the following experiment was carried out. A series of aqueous solutions of H<sub>2</sub>SO<sub>4</sub> and NaOH, ranging in pH from that of M H<sub>2</sub>SO<sub>4</sub> to that of approximately 2 M NaOH, was prepared. The pH of these solutions was accurately determined, and 50 ml. of each solution were treated in a similar manner to that involved in the quinine adsorption. The pH of the resultant series of filtrates was determined for each earth. The same procedure was afterwards repeated by using in each 1 gm. of earth and 250 ml. of solution. The SO<sub>4</sub> content of the four most acidic solutions was determined accurately before and after the fullers' earth treatment. Likewise, the Na content of the four most alkaline solutions was determined before and after the treatment. The differences were attributed to the exchanges having taken place between the earth and the solution. The data obtained are presented in Table I.

It is evident from these data that the pH change during adsorption is due chiefly to the adsorption of the cations, as the SO<sub>4</sub> concentration of the filtrate shows no consistent variation from the original solution, while the concentration of both H and Na ions is decreased. This indicates that the adsorptions of ions by this adsorbent is brought about by an exchange of ions between the adsorbate and the adsorbent, and does not involve the whole molecule. Those ions which combine with the various constituents of the adsorbent to form insoluble products, and are removed from the system by filtering or other mechanical means, are con-

sidered adsorbed. The basic ions removed from the adsorbent by the sulfuric acid, react to form soluble sulfates; consequently no  $\text{SO}_4$  ions are removed from the system when the insoluble adsorbent is removed. Cations like H and Na react to form insoluble silicates, which result in these ions being removed from the system.

This fact becomes more evident when we compare the adsorption of these ions by the electrodialyzed and the untreated earth. The process of electro dialysis will be discussed elsewhere. This treat-

TABLE I.

*Mg. Equivalent of H,  $\text{SO}_4$ , and Na Ions Adsorbed by 1 Gm. of Fullers' Earth.*

Ions.	Approximate concentration of ions.	Mg. equivalent.	
		By untreated earth.	By electrodialyzed earth.
H*	M		
	2	34.80	27.84
	0.2	24.96	14.04
	0.02	1.92	1.26
$\text{SO}_4$ †	0.002	0.32	0.16
	1	0.25	0.27
	0.1	0.05	0.11
	0.01	0.01	0.00
Na†	0.001	0.07	0.04
	2	3.54	9.15
	0.2	2.29	2.80
	0.02	0.45	0.91
	0.002	0.23	0.30

\* Calculated from degree of dissociation and pH change.

† Determined by quantitative method.

ment had little effect upon the adsorption of the anion but brought about a significant alteration in the amounts of both cations investigated. The electrodialyzed earth adsorbed considerably less H ion than the untreated earth, while it adsorbed 2.5 times as many Na ions as a similar weight of earth before treatment. This is to be expected, since by electro dialyzing fullers' earth many of its basic components are replaced by equivalent quantities of H ions, and the resultant product is more nearly saturated with H ions; consequently, less H ions can be adsorbed. This treatment,

as would be expected, has the opposite effect upon the adsorption of Na ions. The electro dialyzed earth, being more nearly depleted of its basic ions, has a greater adsorptive capacity for Na ions than does the untreated earth.

### *Effect of Treating Earth.*

In testing the adsorptive capacity of the different fullers' earths it became evident that certain earths had a much greater adsorption capacity for quinine than other earths. We, like others, became interested to know what factor or factors determined the magnitude of this difference. The first factor investigated was the degree of fineness of the fullers' earth used. A quantity of the earth was sieved by a series of seven sieves ranging from 20 to 300 meshes per inch. The amount of quinine bisulfate adsorbed by each size of particle from an aqueous solution at a pH of 3.30 was determined.

Our first experiences with the different sizes of particles showed a marked decrease in the adsorptive capacity of the larger particles. However, with fullers' earth, which had been previously passed through a 150 mesh sieve, dampened, and allowed to dry in hard lumps, and passed through the same series of sieves no variations in the adsorptive capacity of the various sizes of particles were found. From this it was concluded that the poor adsorption with the larger particles of the original earth was in part due to the large quantity of sand and inert material which naturally would predominate in these fractions, and that the state of division of this adsorbent is of minor importance so far as quinine adsorption is concerned, provided that sufficient time is given for equilibrium to be reached. The poorer adsorption of quinine by the American earths, despite the fact that they were apparently in a finer state of division than the English earths and occupied 1.7 times the volume per unit weight, is a further indication that the size of particle is of relatively small importance in this type of adsorption.

The next factor to be investigated was the effect of previous heat treatment of the earth. 250 gm. of the 150 mesh fullers' earth were divided into five equal parts, four being heated and the fifth remaining unheated. Equal portions of the earth were heated for 30 minutes at approximately the following temperatures:

250°, 500°, 750°, and 1000°. The amount of alkaloid adsorbed by samples of each portion was then determined. It was found that the ability of the four heated portions of earth to adsorb quinine was reduced 36.8, 71.9, 86.6, and 98.7 per cent respectively. Lloyd (7) has attributed such changes in adsorptive capacity to different degrees of hydration. If dehydration is the only change that took place when the above earth was heated, then we believe the differences in the adsorptive capacities of the two types of earth studied can be explained on this basis. It seems more plausible, however, that the decreased adsorption resulted from a conversion of the replaceable cations of the earth into a more stable form by the heating. This view is substantiated by the fact that the iron present in the earth is converted into red oxide by heating the earth. This is evident from the red coloration of the earth, which increases with a decrease in the adsorptive capacity of the heated earth.

The next step was to study the effect of electrodialysis upon the adsorptive capacity of the earths. This treatment ranged from 8 to 32 hours at an E.M.F. of 90 to 140 volts, and with 2 to 0 amperes. When the earth had dialyzed for the desired period of time, it was washed from the dialyzing cell, filtered by suction, and dried in an oven at a temperature not exceeding 60°. The dry material was passed through a 150 mesh sieve and its adsorptive capacity for quinine determined.

The effect of electrodialyzing the English earths against distilled water for 32 hours is shown by a comparison of Curves 1 and 2 (Fig. 2). Curve 1 represents the adsorption of quinine from alcoholic solutions by the original earth, when plotted on the basis of equilibrium H ion concentration. Curve 2 represents similar data obtained from the dialyzed earth. While the general trend of the two curves is very much the same, the electrodialyzed earth adsorbed the maximum quantity of quinine in a more acidic solution than the untreated earth. This is to be expected since the dialysis tended to make the earth more acidic by the removal of the water-soluble electrolyte and at least part of the replaceable bases, which resulted in the shifting of the isoelectric point of the treated earth toward the acid side of the pH range.

Of the factors investigated thus far, heating proved most effective in altering the adsorptive capacity of a given earth. When

an earth has been heated sufficiently to impair its adsorptive capacity seriously, a reddish coloration of the earth resulted. This coloration suggested the presence of iron compounds. Since the English earths which were the most effective as an adsorbent of quinine likewise showed the most color on being heated, this sug-

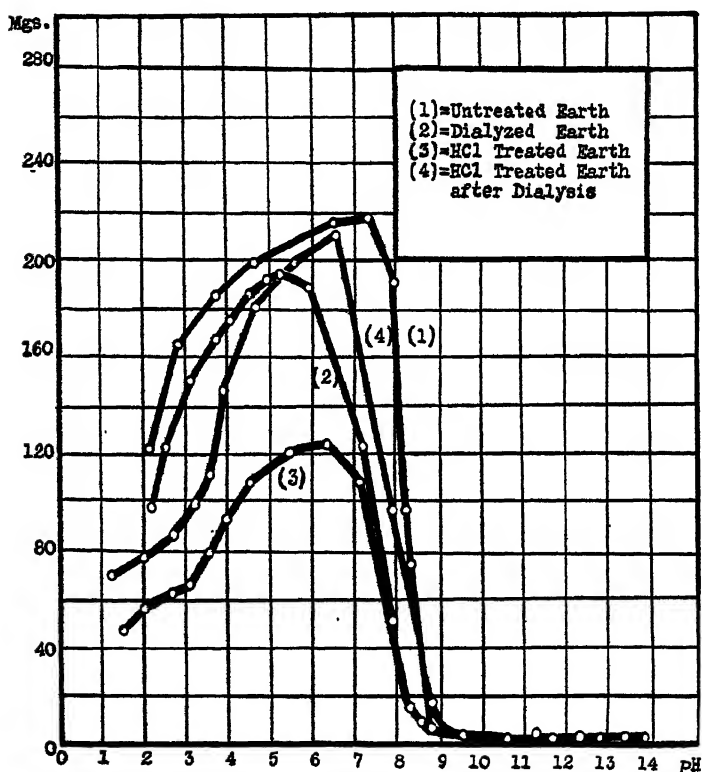


Fig. 2. Mg. quinine bisulfate adsorbed by treated fullers' earth from aqueous alcohol at various equilibrium pH values.

gested the possibility that the iron compounds were related to adsorption of the alkaloid, consequently a sample of English earth was digested in boiling 10 per cent HCl. The earth was filtered out and the treatment repeated for a total of five extractions. The residue was washed with distilled  $H_2O$  until the filtrate was free from chlorides and was then dried at a low temperature. The

dried material was then passed through a 150 mesh sieve and its adsorptive capacity for quinine determined.

Curve 3 (Fig. 2) indicates the adsorption of quinine by the earth after the treatment with the hot acid. There was a loss of about 48 per cent of the original adsorptive capacity of the material. This adsorbent had a much greater effect on the reaction of the alkaline solutions than the original material. The maximum adsorption of quinine occurred in the solution having an initial pH of 10.26 and an equilibrium pH of 5.6. Whether the decreased acidity was due to adsorption of Na ions or to neutralization of Na by adsorbed Cl ions was not determined.

A portion of the acid-treated earth was electrodialed until the diffusible ions were exhausted. Curve 4 (Fig. 2) shows the ability of this material to adsorb quinine. The adsorption by the acid-treated and dialyzed earth was nearly as effective as the adsorption by the original earth. It was noted that during the early stages of the dialyzing a considerable quantity of chlorine was liberated from the anode chamber; this was followed by the appearance of hydrochloric acid in the anode chamber and basic ions in the cathode chamber. This phenomenon suggested the possibility of the replacement of OH groups from the earth by Cl ions during the drastic acid treatment; such a reaction would result in the formation of a chlorosilicate complex and decrease the potential store of exchangeable cations (H ions) in the adsorbent.

In order that a study might be made of the adsorptive properties of the substances removed from the fullers' earth, the combined filtrates and washings were placed in a large evaporating dish and brought to pH 6.8 with NaOH. A reddish gelatinous precipitate formed which was removed, after the solution became cool, by filtering with suction. This red precipitate was washed until the filtrate gave a negative chloride test and then dried. The filtrates and washings were combined and made distinctly alkaline with ammonium hydroxide. A grayish gelatinous precipitate formed, which was treated in a similar manner as the reddish precipitate.

The precipitated  $\text{Fe}_2\text{O}_3$  and  $\text{Al}_2\text{O}_3$  were electrodialed until free of diffusible ions and their adsorptive capacities for quinine determined. There was practically no adsorption of quinine by either adsorbent in the acid solutions and only a very slight adsorption in the alkaline solutions.



*Adsorption of Oxalate.**Experimental Procedure.*

4 gm. of oxalic acid were dissolved in 750 ml. of distilled water, the acidity or alkalinity adjusted to approximately the desired pH

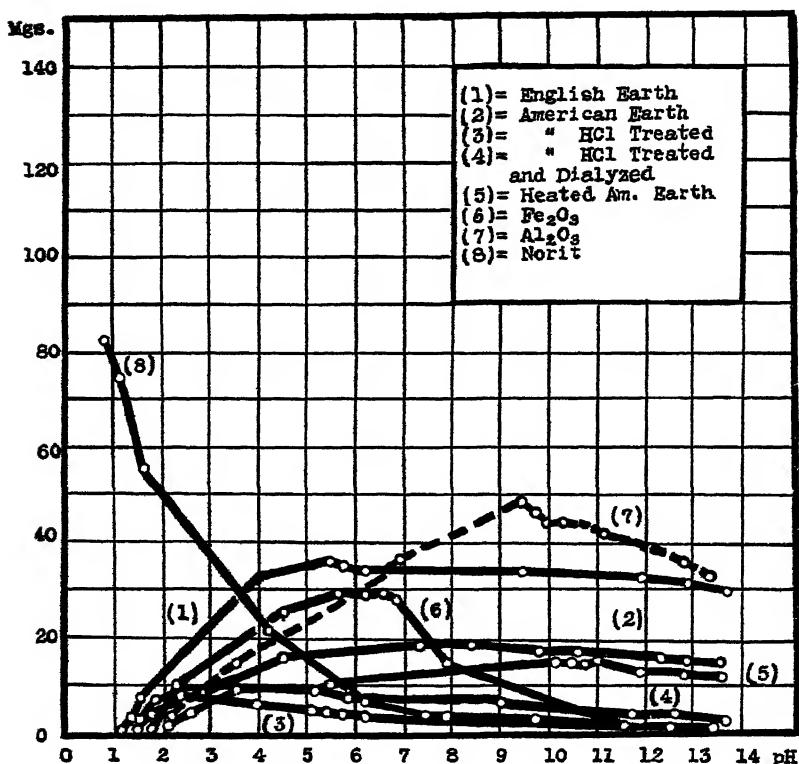


FIG. 3. Mg. of oxalate adsorbed per gm. of adsorbent from aqueous solutions at various equilibrium H ion concentrations.

with  $\text{H}_2\text{SO}_4$  or  $\text{NaOH}$ , and the volume increased to 1000 ml. After the solution was mixed thoroughly, the H ion concentration and the oxalate content were determined. A series of twelve solutions was prepared in this manner, ranging in pH from 0.98 to 13.53 and containing the equivalent of 4 mg. of oxalic acid per ml.

For the adsorption measurement, 50 ml. of solution and 1 gm.

of the adsorbent were placed in a dry 100 ml. flask, shaken for 1 hour, and filtered through a small quantitative filter into a dry receptable. H ion determination was made on a small portion of the filtrate. The unadsorbed oxalate was determined by titrating duplicate 10 ml. portions of the filtrate with standard permanganate solution. Blank determinations were made upon original solutions, by putting them through a similar procedure but omitting the adsorbent.

#### *Effect of H Ion Concentration.*

The effect of H ion concentration upon the adsorption of oxalate ion is illustrated in Fig. 3. Curve 1 shows the adsorption of oxalate from aqueous solutions of various H ion concentrations by English fullers' earth. The maximum adsorption occurred in the region of the neutral point. There was not the sharp decrease in adsorption on either side of this point, which was noted on each side of the optimal pH in the quinine adsorption. There was, however, a pronounced inhibiting action in the solutions more acid than pH 4.0, the adsorption reaching 0 around pH 1.0. The effect of alkaline solutions was less pronounced. Curve 2 represents the adsorption of oxalate ion by American fullers' earth. This adsorbent was less effective than the English earth but the effect of the reaction of the solution upon the adsorption is comparable.

Curve 3 indicates the adsorption of oxalate by the American earth after it had been treated with hot hydrochloric acid, and Curve 4 shows the effect of electro dialyzing the acid-treated earth. It is evident that the acid treatment almost destroyed the power of the earth to adsorb the oxalate ion and that this ability was not restored by the electro dialyzing process.

The effect of heating the American earth for 30 minutes at approximately 1000° is shown in Curve 5. This treatment depressed the adsorption, particularly in the acidic solutions. In the stronger alkaline solutions there was a very slight depression of the adsorption owing to the previous heating of the adsorbent.

Curves 6 and 7 show the adsorption of oxalate by the iron and aluminum preparations which were obtained in the previously described treatment of the fullers' earth with hydrochloric acid. The adsorption by these substances in the acid region was quite similar to that by the original earth from which they came. The

ferric oxide adsorbent showed an optimum adsorption in the region of pH 5.0 to 7.0 and rapidly decreased in efficiency with further increase in alkalinity. The aluminum preparation on the other hand, increased in efficiency until a pH of about 9.5 was reached and decreased very gradually in the more alkaline solutions.

Norit was the most efficient of the substances tested as adsorbents for oxalate. Curve 8 shows that the effect of H ion upon the adsorption of oxalate by norit was quite different from its effect upon the adsorption by the other adsorbents; the most efficient adsorption by norit occurred in the most acidic solutions used and declined rapidly with decrease in H ion concentration.

It seems evident from the data presented that the adsorption of oxalate by fullers' earth depends upon the basic substances contained by the earth and not upon the silicate part of the complex. This is indicated by the greater efficiency of the English earth which analyses showed to contain more basic substances than the American earth. It is further shown by the fact that removal of the bases by acid treatment almost completely destroyed the ability of the earth to adsorb oxalate. Moreover, the oxides of iron and aluminum which were removed from the English earth were found to be active adsorbents of oxalate. It is apparent that the adsorption of oxalate by fullers' earth is simply the formation of insoluble oxalates with certain bases of the adsorbent; anything which favors the formation of these insoluble oxalates will, therefore, increase the apparent adsorption of oxalate ion. The decrease in adsorption of oxalate with increase in the concentration of H ion is apparently a reflection of increasing solubility of the oxalates formed.

#### *Adsorption of Glucose.*

#### *Experimental Procedure.*

A series of aqueous solutions of glucose was prepared, ranging in pH from 0.87 to 12.46 and containing 10 gm. of purified glucose per liter. Solutions of glucose, more alkaline than 12.45, were prepared but were not used because of the destruction which became apparent through the brown coloration which formed. This coloration was not observed in the acidic solutions, or alkaline solutions with a pH less than 12.46, even after they had stood

several days. For the adsorption, 1 gm. of the adsorbent was weighed accurately and placed in a 100 ml. Erlenmeyer flask and 50 cc. of the solution added. The flask was shaken from time to time during a period of 1 hour, and filtered through a small filter into a dry 100 ml. volumetric flask. A small portion of this fil-

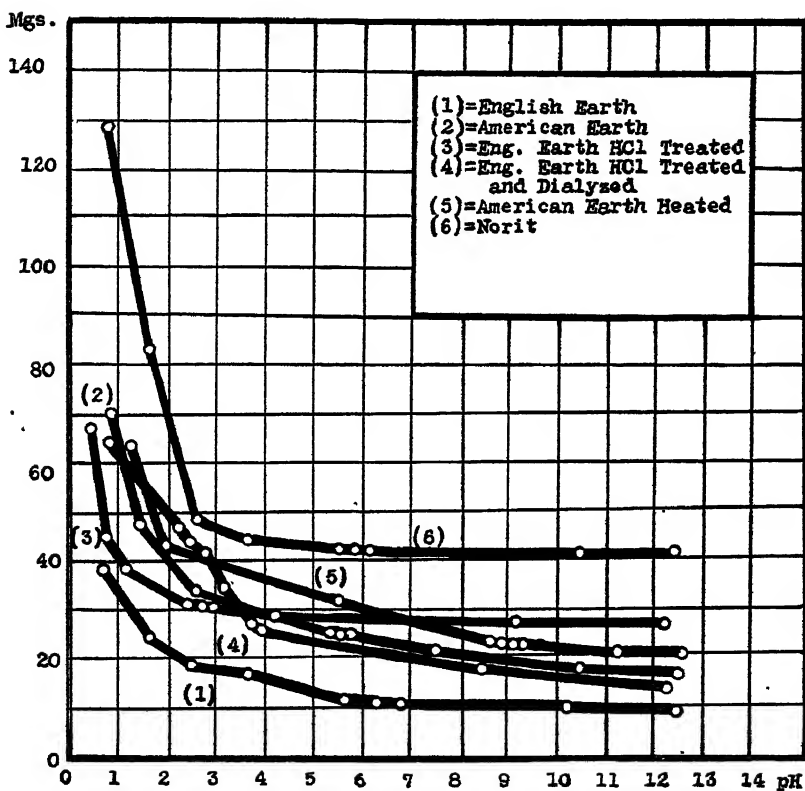


FIG. 4. Mg. of glucose adsorbed per gm. adsorbent from aqueous solutions at various equilibrium H ion concentrations.

trate was used in the pH determination. The unadsorbed glucose was determined by the Benedict (24) method. The amount of glucose adsorbed by a number of adsorbents was determined by this method. Blank determinations were made upon all solutions previous to the treatment with the adsorbents.

*Effect of H Ion Concentration.*

Fig. 4 shows the effect of the H ion concentration on the adsorption of glucose from aqueous solutions by fullers' earth and norit. Curve 1 represents the weight in mg. of the glucose adsorbed by 1 gm. of English earth at the various equilibrium H ion concentrations. The greatest adsorption of this substance was obtained in the most acidic solutions. This is not in agreement with the findings of Hauge and Williaman (23); however, the solutions employed by us were considerably more acidic than the ones used by these investigators. Another factor which might have led to different results is that our solutions contained twice as much glucose as the solution used by Hauge and Williaman. The adsorption of this substance in acidic solutions probably is affected by at least two important factors. The first of these factors is the solubility of glucose in strongly acidic solutions. Since glucose is a slightly acidic substance, its solubility in strongly acidic solutions is less than in water or dilute sodium hydroxide solutions. This decreased solubility in strongly acidic solutions results in a more favorable condition for adsorption. The second factor is the effect of acid on the surface tension of glucose solution. Glucose, when dissolved in distilled water, has very little effect upon its surface tension, but the same amount of glucose dissolved in dilute sulfuric acid has a very outstanding effect upon the surface tension of the resulting solution. When 10 gm. of glucose were dissolved in 1 liter of distilled water at 26°, its surface tension was raised 0.5 dyne, while the same weight of glucose dissolved in an equal volume of M sulfuric acid at the same temperature resulted in the lowering of the surface tension by 15.8 dynes. This lowering of surface tension creates a condition most favorable for adsorption.

Curve 2 shows the adsorption of glucose by a sample of American earth. This earth was in a much finer state of division than the English earth, and consequently had a greater specific surface. This being true, a greater adsorption of a non-polar substance is to be expected. Curve 3 shows the adsorption by a sample of the English earth after having received the hydrochloric acid treatment. Such a treatment always resulted in a more acidic product and in a higher degree of fineness than the original earth.

Both of these alterations are evident from the adsorption curve. Curve 4 shows the adsorption by the acid-treated earth after being electrodialyzed. It is apparent that electrodialysis did not affect the adsorption by this substance other than to reduce its acidity.

Curve 5 shows the adsorption by the American earth after being heated. Evidently, the only detrimental effect of heating the earth upon the adsorption of glucose is that the resultant product is always more alkaline than the unheated earth. This tends to shift the pH of the solution farther toward the alkaline side which results in a slight decrease in adsorption in the more acidic solutions.

Curve 6 shows the adsorption of glucose from the same series of solutions by norit. It is evident from this curve that the adsorption by this substance is affected by the same factors as those affecting the adsorption of glucose by fullers' earth. The greater adsorption by this adsorbent is evidently due to its greater specific surface.

#### DISCUSSION.

It is recognized that the data presented in the preceding pages are by no means complete. Nevertheless, the results obtained have afforded helpful suggestions for the control of conditions in the adsorption of the active factors of the vitamin B complex. Some of the results likewise seem to have general application to adsorption problems.

The most important factor affecting the efficacy of fullers' earth as an adsorbent is the nature of the earth itself. The problem why one variety of earth is an active adsorbent and another variety practically worthless, has never been completely solved. The present studies have shown, however, that the relative efficiency of different earths varies with the character of the substance to be adsorbed. The ability of an earth to adsorb alkaloids or other basic substances probably depends upon the ability of the silicate group of the complex to ionize and bind cations. This property no doubt is closely related to the degree of hydration which was pointed out by Lloyd as a requisite of adsorption by this agent. The adsorptive capacity of an earth for anions, on the other hand, is a direct result of the formation of insoluble compounds of the anions with the basic ions of the adsorbent. The efficiency of an earth in this type of adsorption is measured,

therefore, by its content of bases which are capable of forming insoluble complexes with a given anion. The adsorption of a non-electrolyte is apparently not related to the ionization of the earth but is a function of the specific surface of the adsorbent. The English earths are apparently more efficient than the American earths for the adsorption of electrolytes; the American earths, however, excel as adsorbents for non-electrolytes of the glucose type. The English earths used in these studies weigh about 1.75 times as much per unit volume as the American earths; this indicates a smaller specific surface for the former and probably explains their less efficient adsorption of glucose.

The effect of various treatments upon the adsorptive capacity of fullers' earth, likewise, varies with the type of substance to be adsorbed. Heating of an earth for 30 minutes at about  $1000^{\circ}$  almost completely destroys the ability to adsorb quinine; this treatment slightly impairs the ability to adsorb oxalate; the ability to adsorb glucose, however, is not affected significantly by this degree of heating. The heating no doubt dehydrates the salts of silicic acid and thus results in the formation of the stable oxide which cannot be hydrated again under the conditions in which it was tried as an adsorbent.

A surprising result is that electrodialysis of the English earth decreases its efficiency as an adsorbent of quinine. Since the basic substances in the natural earth do not adsorb quinine, it seems logical to expect that their removal would increase the efficiency of the purified residue. This was not found to be true but the explanation is not apparent.

Treatment of English fullers' earth with hot hydrochloric acid greatly reduces its ability to adsorb quinine, almost completely destroys its ability to adsorb oxalate, but increases its efficiency as an adsorbent for glucose. This treatment removes nearly all the bases from the silicate complex and replaces them with hydrogen. There is a further possibility that the treatment is sufficiently drastic to cause the silicic acid thus formed to give up some OH ions for which Cl ions are substituted. In each group thus formed the possibility of an exchange of H ions for any other cation would be lost. The escape of chlorine gas at the anode and the restoration of the efficiency of the acid-treated earth upon electrodialysis seem significant in this connection. Since the bases

in the crude fullers' earth are responsible for the adsorption of oxalate, it is evident that their removal by any treatment would prevent the adsorption of this ion. The increased adsorption of glucose is probably due largely to an increased dispersion of the adsorbent and to the fact that the acid-treated adsorbent has less tendency to decrease the H ion concentration of the solutions.

One of the most important controllable factors in adsorption by fullers' earth is the H ion concentration of the solution in which the adsorption takes place. While the present studies have been definitely related to the concentration of H ion, the adsorption really is influenced by the concentration and relative activity of all the ions in the system. If fullers' earth is treated with a solution containing a mineral acid, the bases are removed from the earth and replaced by their equivalent of hydrogen until equilibrium is established. The bases are removed in the order of their position in the electromotive series; the higher the element in the series, the more easily is it replaced. If the process is continued far enough, all of the basic ions will be replaced by H ions. Now if the acid adsorbent is placed in an alkaline solution the reverse reaction occurs; the basic ions replace the H ions until an equilibrium is established. On the acid side of what may be termed the isoelectric point of the earth the tendency is toward the adsorption of the weaker cations but on the alkaline side of this point the tendency is toward the adsorption of the stronger cations; the reaction is favored in the former case by the formation of the weakly ionized silicic acid and in the latter case by the formation of  $H_2O$  as one of the end-products.

It is evident, therefore, that a weak basic ion can more readily compete with H ions in an acid medium than can a strong basic ion; hence the weaker the cation, the greater is the concentration of H ion favoring its optimal adsorption.

The adsorption of anions by fullers' earth may likewise be considered as a competition between the various cations of the system, the same factors affecting the pairing of ions as are operative in the adsorption of cations. It is evident that the maximum adsorption of a given anion will occur under conditions in which those cations of the system, which form soluble compounds with the given anion, replace from the fullers' earth those cations which form insoluble compounds with the anion to be adsorbed. The



formation of these insoluble compounds is obviously a requisite of adsorption.

The adsorption of non-electrolytes apparently is not affected by the ionization of the adsorbent. The H ion concentration of the solution, nevertheless, may play an important part in the adsorption of such substances. In this type of adsorption the importance of solubility of the substance to be adsorbed and the effect of viscosity and surface tension of the solution are at a maximum. In so far as H ion concentration affects these phenomena, it will also affect the adsorption of substances like glucose.

The optimal adsorption of quinine and of glucose by norit occurs at the same H ion concentration which favors their optimal adsorption by fullers' earth. The effect of H ion concentration upon the adsorption of oxalate is quite different for the two adsorbents. The adsorption of this compound by norit gradually decreases from pH 0.98 to pH 7.0 and is almost negligible throughout the alkaline range. It is possible that the adsorption of quinine by norit is identical in nature with its adsorption by fullers' earth; in the case of norit there probably is an exchange of cations from inorganic impurities in the adsorbent for quinine ions. As a consequence of the low inorganic content of norit, it is a much poorer adsorbent for quinine than is fullers' earth. Norit is, however, a better adsorbent for oxalate than is fullers' earth; this is especially true at the higher H ion concentrations. It is apparent that norit, unlike the bases of fullers' earth, can form compounds with oxalate which are insoluble in the most acidic solutions used in these tests.

#### SUMMARY.

1. A study of the effect of certain factors upon the adsorption of quinine, oxalate, and glucose by fullers' earth and norit has been made.

2. Different fullers' earths have different adsorptive capacities because of differences in both composition and state of division.

3. H ion concentration has been found to play an important rôle in the adsorption of electrolytes, and under certain conditions, it also affects the adsorption of non-electrolytes.

4. Degree of dispersion of adsorbent is more important in apolar adsorption than in polar adsorption.

5. By heating fullers' earth at a red heat for 30 minutes, its adsorptive capacity for both oxalate and glucose is only slightly altered.

6. The electro dialyzing of fullers' earth slightly reduces its adsorptive capacity for quinine.

7. Fullers' earth which has been previously digested with hot mineral acid, adsorbs only one-half as much quinine as the untreated earth, while it adsorbs only one-sixth as much oxalate as before the treatment. Such treatment has little effect upon the adsorption of glucose by this adsorbent.

8. Electrodialysis of the acid-treated earth restores much of its quinine adsorptive capacity but has little effect upon the adsorption of either oxalate or glucose.

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## THE EFFECT OF HYDROGEN ION CONCENTRATION UPON ADSORPTION OF THE ACTIVE FACTORS OF VITAMIN B COMPLEX BY FULLERS' EARTH.\*

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Fullers' earth has been used as an adsorbent for vitamin B by several investigators (1-7). In general the practice has been to adsorb the vitamin from acidulated extracts of various plant substances. Either the activated fullers' earth was then fed as a source of the vitamin, or the adsorbed substance was displaced from the adsorbent by treatment with alkaline solutions of varying strength. The methods have been more or less empiric and the reactions of the solutions used in both the adsorption and the elution processes have varied within rather wide limits. This has to a large extent been due to a lack of sufficient data regarding the influence of H ion concentration upon the adsorption process. Levene and van der Hoeven (3) have concluded that the H ion concentration for the optimal adsorption of the vitamin B complex by Lloyd's reagent as indicated by the rat growth method is at pH 4.0. Their results, however, afford no idea of any possible differences in the adsorption of the separate factors of the complex. Kinnorsley and Peters (8) have reported that the optimal adsorption of the B-P factor ("torulin") by charcoal occurs at pH 7.0 and that the active substance is not adsorbed at pH 2.5. These investigators (9) reported unsatisfactory adsorption of the active factor upon fullers' earth but they do not report the reaction at which the adsorption was attempted.

Previous studies in this laboratory (6, 7) have shown promising results from the use of fullers' earth as a selective adsorbent for

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the B-P factor. It was thought possible that a strict control of hydrogen ion concentration might further increase the selective action, particularly if the two factors should have different optima. In the accompanying paper, Guerrant and Salmon (10) report the effect of H ion concentration upon the adsorption of an organic cation, anion, and also of a non-electrolyte by fullers' earth. These studies emphasize further the relation between reaction of the solution and efficiency of adsorption by fullers' earth.

A study of the effect of H ion concentration upon the adsorption of the B-P and the P-P factors has yielded results which are deemed important in the development of methods for concentrating these factors. An account of the study is presented in the following pages.

#### *Preparation of Adsorbates from Extract of Maize.*

White maize of the current year's crop was dried at 45-50° and finely ground. The ground material was placed in large percolators and extracted at room temperature with 80 per cent (by weight) alcohol. The percolation was continued until 225 liters of extract were obtained from 150 kilos of maize. This extract was then reduced by vacuum distillation to a volume of about 15 liters, allowed to cool, and the mass of precipitated protein was removed. The protein was kneaded thoroughly to remove as much as possible of the occluded extract which was poured off and the protein again kneaded in three successive portions of water. The washings were added to the extract and the total volume made up to 21.5 liters with H<sub>2</sub>O. The volume was then reduced as before to about 15 liters to insure complete removal of the alcohol. The extract was kept in an electric refrigerator for about 48 hours and was then filtered by suction and diluted to 16.5 liters. The filtrate was a clear yellowish fluid with a surface tension of 55 dynes, a pH of 4.70, and a sp. gr. of 1.005. This solution was divided into ten portions, the first four representing 10 kilos of maize each, the next two 15 kilos each, and last four 20 kilos each. The reaction of the various portions was adjusted with H<sub>2</sub>SO<sub>4</sub> or NaOH to the following respective values: pH = 0.54, 1.56, 2.73, 4.16, 5.64, 6.81, 8.15, 9.59, 10.57, 11.89. All pH determinations were made with the calomel electrode. The final volume of each portion was kept proportional to the amount

of original maize it represented. After the pH adjustments had been made, surface tension, viscosity, and specific gravity measurements were taken. The various portions were then treated with 3 gm. of English fullers' earth per kilo of maize, each solution being stirred vigorously at 5 minute intervals for a period of 2 hours. The adsorbates (activated solids) were then removed on Buchner funnels and washed twice with 25 ml. of 80 per cent alcohol per 30 gm. of solid and twice with the same amount of 93 per cent alcohol. The various portions of the adsorbate were partially dried at room temperature, pulverized, and placed in vacuum desiccators until completely dry.

The washings from the adsorbates were freed of alcohol by vacuum distillation and returned to their respective filtrates. The pH, surface tension, and viscosity were again determined on each filtrate. After the alkaline filtrates were made distinctly acid to litmus, all filtrates were allowed to stand overnight. The reaction of each filtrate was then adjusted to approximately pH 4.0 and the concentration of  $\text{Na}_2\text{SO}_4$  equalized by the addition of solid  $\text{Na}_2\text{SO}_4$  where necessary. Each solution was again treated with the same amount of fullers' earth as before, the procedure being identical for the two treatments, except the pH of the solutions which had been readjusted as mentioned.

#### *Preparation of Adsorbates from Extract of Yeast.*

The yeast was extracted in portions of 2.5 to 5.0 kilos and the concentrated extract divided into four or five portions which were treated with fuller's earth at different H ion concentrations. Since the procedure was uniform for all the preparations, the method for one lot is described as typical of the series.

*Yeast 1.*—8 liters of  $\text{H}_2\text{O}$  acidulated with 0.75 per cent by volume of glacial acetic acid were heated to  $95^\circ$ . A 2.5 kilo portion of dry bakers' yeast was then stirred in and the mass thoroughly agitated. The mixture was allowed to cool to  $60^\circ$  and then received an addition of 12 liters of 93 per cent alcohol which flocculated the protein and facilitated the filtering process. The coagulate was filtered out on Buchner funnels and again extracted with 4 liters of warm 51 per cent alcohol. The combined filtrates were concentrated by vacuum distillation to 6.6 liters. After having stood in a refrigerator overnight the concentrate

was filtered and divided into four aliquots. The pH adjustments were made by the use of  $\text{H}_2\text{SO}_4$  or  $\text{NaOH}$ , and 160 gm. of American fullers' earth per kilo of yeast were added. The mixtures were agitated intermittently for 2 hours. The adsorbates were then filtered out and each portion washed with four 25 ml. portions of  $\text{H}_2\text{O}$  and one 50 ml. portion of 93 per cent alcohol. They were then dried as in the maize preparations.

The washings were combined with their corresponding filtrates and each portion readjusted to the original pH of the concentrated extract. Each portion was then concentrated by vacuum distillation. When the volume had been reduced to 100 to 200 ml., sufficient alcohol was added to give a 51 per cent alcoholic solution (by weight). The precipitate of inorganic salts was filtered out and washed with three 50 ml. portions of 51 per cent alcohol. The filtrate and washings were again concentrated as before. When the solution reached a sirupy consistency it was poured upon 0.32 gm. of Argo corn-starch per gm. of yeast represented. The mixture was dried in an oven at  $50-55^\circ$ , ground, sieved, and stored in glass containers. These evaporated filtrates are the residues which will be referred to in the growth experiments.

*Yeast 2.*—The procedure was the same as for Yeast 1 except that only fifteen-seventeenths as much fullers' earth was used per unit of yeast. This variation was made because previous tests had shown Yeast 2 to be only fifteen-seventeenths as potent in B-P activity as Yeast 1.

#### *Effect of pH upon Adsorption of the B-P Factor.*

The B-P activity of the adsorbates from the maize and the yeast preparations was determined by establishing the minimum dosage required to protect completely pigeons weighing 300 to 350 gm. The essentials of the method have been described elsewhere (6). In the present experiments each bird received a total of 16.0 gm. of the basal diet per day. The adsorbates from the corn preparations were fed in double doses on alternate days but those from the yeast preparations, being much less potent, were fed daily.

The effect of pH upon adsorption of the B-P factor is illustrated by Curves A and B, Fig. 1. On the basis of the pH of the extract before adsorption, the optimal adsorption occurred at pH 2.73.

The protective dose of the resulting adsorbate was 0.0525 gm. per day. Thus 100 gm. of the solid carried 1904 protective doses, which is about 14 per cent less than the sample of earth used will

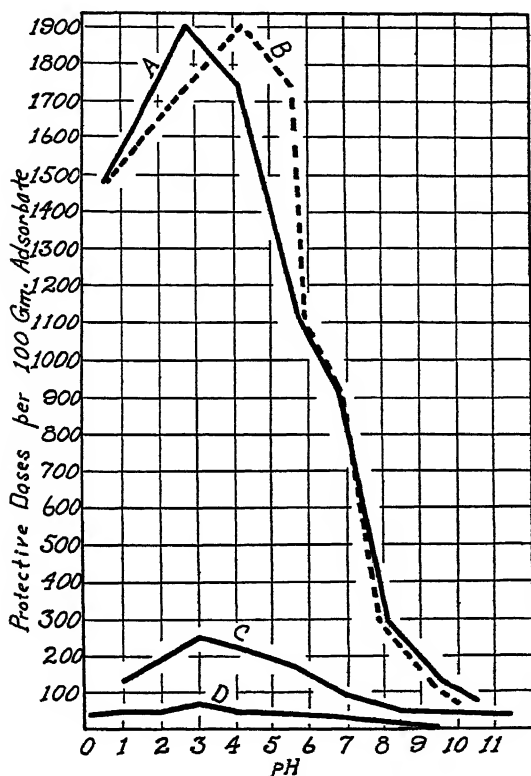


FIG. 1. Effect of pH upon adsorption of B-P factor. Curve A, protective doses per 100 gm. of adsorbates from maize, based on pH of extracts before adsorption. Curve B, same except based on pH of filtrates after adsorption. Curve C, protective doses per 100 gm. of adsorbates from Yeast 1, pH before adsorption. Curve D, protective doses per 100 gm. of adsorbates from Yeast 2, pH before adsorption.

carry when saturated with the B-P factor. The adsorption at pH 0.54 (before adsorption) was 77 per cent; at pH 1.56, 87 per cent; at pH 4.16, 91 per cent; and at pH 5.64, 58 per cent of the optimum and continued to decrease rapidly as the alkalinity in-



creased. In the acid solutions there was apparently an exchange of H ions for basic ions from the fullers' earth and a consequent shift of the pH of the solution toward the neutral point. As a result of this the extract at pH 2.73 before adsorption showed a value of pH 4.2 after adsorption while the one at pH 4.6 shifted to pH 5.6. The pH changes of the other extracts were of considerably less magnitude. The data indicate that there is a zone of maximum adsorption between pH 3.0 and pH 5.5 and that the optimal adsorption may be obtained by holding the reaction constant at pH 4.0 during the adsorption process or by having the solution sufficiently acid that the reaction is not raised above pH 4.0 during this process.

In order to determine whether any of the B-P factor was destroyed by exposure to the alkali, the B-P activity of several of the adsorbates from the second adsorption was determined. There was evidence of some inactivation of the B-P factor beginning at pH 6.81 and increasing significantly throughout the alkaline range. The poor initial adsorption in the alkaline solutions could not be explained on the basis of destruction, since at equilibrium pH 10.0 in the initial adsorption only 80 protective doses of B-P factor per 100 gm. of adsorbent were removed, yet the second adsorption showed that 834 adsorbable protective doses remained in the filtrate from the initial adsorption. Nevertheless, this indicates a loss of 977 protective doses or 51 per cent of the original adsorbable substance, if it is assumed that the sodium sulfate did not decrease the efficiency of the second adsorption. A loss of 51 per cent of the activity by such a short exposure to a weak alkaline solution is quite serious in view of the fact that alkaline solutions seem to offer the best medium for replacement of the adsorbed substance from the fullers' earth. It emphasizes the importance of using a weak alkaline solution and the shortest possible exposure to the alkali in the replacement process.

The optimal adsorption of B-P factor from the yeast extracts was obtained in the extracts having a reaction of about pH 3.00 before adsorption (Curves C and D, Fig. 1). (Unfortunately determinations of pH after adsorption were made on only a few of the yeast extracts.) The trend of the adsorption curves for the yeast extracts is very similar to that of the maize extract. The total adsorption at the optimal pH and the differences in the

amount of the B-P factor adsorbed at different pH values are very much less, however, than in the case of the maize preparations. These differences may be explained in part by the large amount of fullers' earth used for the yeast extracts. Previous tests have shown that the sample of fullers' earth used for the yeast preparations is able to adsorb 1000 protective doses per 100 gm. of earth. The most efficient activated solid from the yeast preparation (prepared from Yeast 1 at pH 3.06) carried only 250 protective doses. It is apparent that the concentration of B-P factor in the yeast extract was only sufficient for 25 per cent of saturation of the fullers' earth at the optimal pH. The effect of pH on adsorption of the B-P factor doubtless would have been more pronounced with sufficient of the active factor in solution for complete saturation of the solid at the optimal pH. The results from Yeast 2 are in agreement with this view. The most active solid (adsorption pH = 3.0) from this sample contained only 71 protective doses and the adsorption curve for this sample is even flatter than the curve for Yeast 1.

A comparison of the behavior of the two yeasts shows some rather striking differences. The amount of Yeast 1 used for 100 gm. of fullers' earth carried 416 protective doses of B-P factor. From this amount 250 protective doses per 100 gm. of solid were recovered at pH 3.0, giving a recovery of 60 per cent of the original activity of the yeast. The amount of Yeast 2 used per 100 gm. of fullers' earth represented the same number of protective doses. Nevertheless, only 71 protective doses, 17.6 per cent of the original activity, were recovered at the optimal pH. Tests with rats failed to demonstrate any B-P factor remaining in the residue (filtrate) from the pH 3.0 adsorption of Yeast 2 but did indicate the presence of slight B-P activity in the corresponding residue from Yeast 1. It seems probable that the discrepancy was due to incomplete extraction of the active material from Yeast 2.

#### *Effect of pH upon Adsorption of the P-P Factor.*

The method of determining the P-P activity of the various preparations from the yeast was essentially the same as used in previous tests in this laboratory (6, 7). Rats (45 to 50 gm.) received Diet 2 B for a preliminary depletion period of 2 weeks. They then received 0.03 gm. per day of B-P Solid 25 as a source

of the B-P factor. This was prepared from an alcoholic extract of maize by the method described in the preceding pages, except that the concentrated extract was adjusted to pH 4.0 and treated with 2.5 gm. of English fullers' earth per kilo of corn. This adsorbate in doses of 0.03 gm. daily furnishes sufficient B-P factor for growth but an insufficiency of P-P factor either to produce growth or to prevent the onset of characteristic symptoms of pellagra. In addition to Diet 2 B supplemented by B-P Solid 25, daily doses of 0.20 gm. of adsorbate from the yeast or 0.25 gm. of the residue (filtrate remaining after adsorption) were fed to each rat. These amounts, even of the most active preparations used, were not adequate to support an optimum rate of growth; hence, any variations in activity would be more readily apparent.

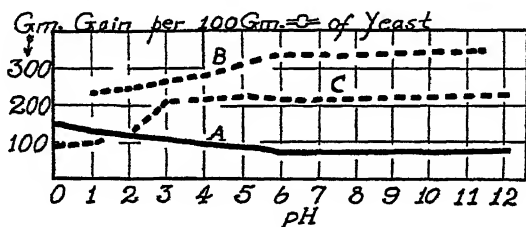


FIG. 2. Effect of pH upon adsorption of P-P factor. Curve A, gain in gm. per 100 gm. equivalent of yeast from adsorbates from Yeasts 1 and 2. Curve B, gain in gm. per 100 gm. equivalent of yeast from residues (filtrates) of Yeast 1. Curve C, same except for Yeast 2.

In order that the P-P activity of the adsorbates and the residues might be compared, the amount of growth produced in 4 weeks by the various preparations is reported on the basis of gain in gm. per 100 gm. equivalent of yeast. The results are shown in Fig. 2. Since the gains produced by the adsorbates from Yeast 1 and Yeast 2 at corresponding pH values were very similar, the results are combined for Curve A.

The most acid solution used, having a value of pH 0.08 before adsorption, yielded the adsorbate with the maximum P-P activity. The amount of active substance apparently decreased gradually from this point to pH 6.3 and remained approximately constant from the latter point to pH 12.07, the most alkaline solution used. It seemed that the P-P factor adsorbed throughout the alkaline range was about one-half that adsorbed at pH 0.08.

The activity of all of the residues was not determined; however, a sufficient number was tested to confirm the findings on the adsorbates. It is evident that the filtrates from the adsorptions in the acid region were significantly less potent in their growth-promoting action than were those from the adsorptions in the alkaline region. A comparison of Curves B and C, Fig. 2, shows that a much greater amount of P-P factor was extracted from Yeast 1 than from Yeast 2. The most potent residues from the alkaline adsorption of extracts from Yeast 1 produced about 340 gm. gain per 100 gm. equivalent of yeast. On the other hand the most active residue from the preparations of Yeast 2 produced only 220 gm. gain per 100 gm. equivalent.

Although the greater activity of the residues from Yeast 1 indicates that there was a notably higher concentration of the P-P factor in the extracts from Yeast 1 than from Yeast 2, this difference was not reflected in the activity of the adsorbates prepared from the two sources. It seems, then, that the potency of the adsorbates was limited by the ability of the fullers' earth to adsorb the P-P factor. This indicates that fullers' earth is not an efficient adsorbent for the P-P factor and suggests the desirability of a better adsorbent. Further work along this line is contemplated.

#### DISCUSSION.

It is apparent that the reaction of the solution determines to a large extent the efficiency of adsorption of either the B-P factor or the P-P factor by fullers' earth. There is a significant difference, however, in the effect of pH upon the adsorption of the two factors. The optimal adsorption of the B-P factor from extracts of yeast or maize was obtained in a zone between pH 3.0 and pH 5.5 (equilibrium pH) with the optimal point at about pH 4.0. From this zone there was a gradual decrease in adsorption with increase in acidity and a rather rapid decrease with increase in alkalinity. The adsorption practically reached zero at about pH 11.0. On the other hand the optimal adsorption of the P-P factor occurred at pH 0.08 the most acid solution tested. There was a gradual decrease in adsorption to pH 6.3, from which point the adsorption remained practically constant throughout the alkaline range of the tests.

It is interesting to compare the adsorption of the two factors with the adsorption of quinine and of glucose as reported by Guerant and Salmon (10). The effect of pH upon adsorption of the B-P factor is very similar to its effect upon the adsorption of quinine; again in the adsorption of the P-P factor and of glucose, there is a comparable effect of pH. Another similarity in the relative amounts of quinine and of glucose adsorbed is found in the apparent relative adsorption of the B-P and the P-P factors. Although it is not possible to determine the absolute amounts of the two factors adsorbed, there are reasons for believing that the adsorption of B-P factor by fullers' earth is much greater than the adsorption of P-P factor. One indication of this is the relative completeness of the removal of the two factors from solution, it being far more difficult to deplete an extract of its P-P activity than of its B-P activity. Again the efficiency of the respective adsorbates seems to indicate a greater adsorption of the B-P factor: it is possible to prepare an adsorbate which has many times the B-P activity of the most potent natural sources of this factor; in contrast with this, the adsorbates from yeast extracts as prepared in these tests were apparently not as potent sources of the P-P factor as the yeast from which they were prepared. These differences seem to suggest phenomena associated in the one case with the polar and in the other with the apolar type of adsorption.

The sensitivity of the B-P factor to an alkaline reaction indicates the importance of using a weakly alkaline eluent in the process of replacing the active factor from the adsorbate. Although it is not certain that the adsorption is reversible, it seems probable that a satisfactory recovery of the active substance may be effected by the use of an eluent having a reaction of pH 8.0 to pH 9.0.

In the above studies the P-P factor has been considered as dual in nature, having both a growth-stimulating and a pellagra-preventing action. It is by no means certain, however, that both properties are inherent in a single substance. An increasing accumulation of evidence seems to indicate that at least two substances are involved in this dual function of the P-P fraction. This introduces the possibility that the adsorption of the P-P factor as determined in these experiments represents a composite

adsorption of more than one active substance. Nevertheless, the data are submitted with the belief that they establish a basis for a more efficient utilization of fullers' earth in the separation and concentration of the components of the vitamin B complex.

#### SUMMARY.

1. The maximum adsorption of the B-P factor by fullers' earth was obtained in a zone between pH 3.0 and pH 5.5 (equilibrium reaction) with the optimal point at pH 4.0.

2. The adsorption of the B-P factor diminished rapidly as the alkalinity increased beyond pH 5.5, becoming negligible at pH 9.0 and practically zero at pH 11.0.

3. There was evidence of significant inactivation of the B-P factor in the alkaline solutions.

4. The maximum adsorption of the P-P factor by fullers' earth occurred at pH 0.08, the most acid solution tested.

5. The adsorption of the P-P factor decreased gradually to a minimum at pH 6.3 and thence remained approximately constant to pH 12.07, the most alkaline solution tested.

6. Fullers' earth seemed to be a more efficient adsorbent for the B-P factor than for the P-P factor.

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# THE CHEMISTRY OF JAFFE'S REACTION FOR CREATININE.

## V. THE ISOLATION OF THE RED COMPOUND.\*

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In a recent number of this *Journal* (1), there was described a compound of 2 molecules of creatinine, 1 of picric acid, 3 of sodium hydroxide, and 3 of water. This was originally obtained by adding a solution of creatinine picrate in sodium hydroxide to absolute alcohol. Better yields were obtained in later experiments in which an additional molar equivalent of creatinine was used. The creatinine in this compound was found to be present in a form that does not give Jaffe's reaction. The full color value of the creatinine present could be obtained only after solution of the compound, addition of acetic acid, and heating the solution to boiling or allowing it to stand at room temperature for at least an hour.

It had previously (2) been found that, although only 1 mol of picric acid entered into the reaction, the maximum color in Jaffe's reaction was not obtained unless at least 2 mols of picric acid were present. It was, therefore, believed to be of interest to ascertain what might be precipitated by running into alcohol an alkaline mixture containing 2 mols of picric acid for each mol of creatinine. The first preparation yielded a red precipitate which, upon analysis, appeared to be a mixture of the compound previously described with a new one containing 1 molecule of creatinine, 1 of picric acid, and 2 of sodium hydroxide. By using a little more picric acid (2.5 or 3 mols) and by avoiding the use of too great an excess of sodium hydroxide, the new compound was obtained in a nearly pure condition.

\*Read before the Section of Organic Chemistry, American Chemical Society, Swampscott, Mass., September, 1928.



After drying *in vacuo*, the substance forms a brilliant red, hygroscopic powder.

When dissolved in water and diluted to contain 10 mg. of creatinine per 500 cc. and compared in a colorimeter with 0.5 N  $K_2Cr_2O_7$ , as in Folin's original method for the determination of creatinine (3), the color obtained corresponds to only about 20 per cent creatinine instead of the calculated 26.8 per cent. But this is probably due to dissociation, for, if the solution of the new compound in 10 cc. of water is treated with a mixture of 15 cc. of

TABLE I.

*Analyses of the New Compound of Creatinine, Picric Acid, and Sodium (or Lead) Hydroxide.*

	Picric acid.	Nitrogen.	Sodium.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated for a compound of 1 molecule of creatinine, 1 of picric acid, 2 of NaOH....	54.2	19.9	10.9
Same with 0.5 mol $H_2O$ .....	53.1	19.5	10.7
Preparation 12.....	51.6	19.6	10.8
“ 13.....	53.5	19.5	11.1
“ 20.....	52.5	20.3	11.5
			Lead.
Calculated for a compound of 1 molecule of creatinine, 1 of picric acid, 2 of lead hydroxide, 2 of $H_2O$ .....	26.6	9.76	48.1
From Preparation 13.....	24.3	9.18*	51.7

\* 9.15 per cent when first dissolved in dilute acetic acid; 9.20 per cent when dissolved in hot 67 per cent  $H_2SO_4$ .

1 per cent picric acid and 5 cc. of 10 per cent sodium hydroxide and is then immediately diluted to 500 cc. the full color value is obtained. It is not necessary to wait 10 minutes for the color to develop.

There can be no doubt, therefore, that it is the formation of this compound that is responsible for the red color of Jaffe's reaction. This does not altogether contradict the view previously expressed (2) that it is the formation of the red tautomer of creatinine picrate that is responsible for the production of the color, for the new compound may be regarded as a compound of the red tautomer

with 2 mols of sodium hydroxide. Treatment of fairly concentrated solutions of the new compound with hydrochloric acid results in precipitation of the red tautomer.

A solution of the new compound gives a red precipitate when treated with basic lead acetate solution. When filtered, washed, and dried over  $H_2SO_4$ , the composition of this precipitate agrees quite closely with that calculated for a compound of 1 molecule of creatinine, 1 of picric acid, 2 of lead hydroxide, and 2 of water. As in the case of the dicreatinine compounds, both the sodium and the lead compound contain more base than the formulæ would require. With the high equivalent weight of lead, this results in a decided effect on the creatinine and picric acid content.

That the nature of the combination between picric acid and creatinine in the new compounds is quite different from that in the dicreatinine compounds, is shown not only by the difference in the behavior of the creatinine but also by differences in the behavior of the picric acid. Analyses for nitrogen in the new lead compound give the same value when the substance is treated directly with hot 67 per cent sulfuric acid as when it is first treated with dilute acetic acid. Moreover, there is no marked destruction of the picric acid when a solution of the sodium compound is treated with dilute hydrochloric acid.

#### EXPERIMENTAL.

The analytical methods were those previously employed (1).

#### *Preparation.*

*Details of Preparation 13.*—To a solution of 17.2 gm. (0.075 mols) of picric acid in 300 cc. of boiling water, there were added 3.39 gm. (0.03 mols) of creatinine. After the solution had been cooled to 15°, 30 cc. of 10 per cent NaOH were added from a pipette, the mixture being stirred constantly. The red solution was decanted from the undissolved material, which was now treated with 30 cc. of 2 per cent NaOH. Complete solution was now effected, and the two solutions were mixed and allowed to stand for 15 minutes. Some sodium picrate separated. The mixture was filtered, the filtrate being received in 3000 cc. of absolute alcohol and stirred constantly. The red precipitate obtained was filtered on a

hardened paper, with suction, washed with alcohol, and dried over sulfuric acid. The yield was 3.1 gm. or 25 per cent of the calculated.

*Recovery of Creatinine Picrate from the Sodium Compound.*

1.49 gm. of Preparation 20 were dissolved in 35 cc. of water and the solution was then acidified with acetic acid. The precipitate that formed was partly red and partly yellow. After standing overnight, it was all yellow. The crystals were filtered out and recrystallized from hot water. They melted at  $222^{\circ}$  and contained 67.4 per cent picric acid and 24.3 per cent nitrogen. The values calculated for creatinine picrate are 67.0 per cent picric acid and 24.6 per cent nitrogen. The yield was 0.89 gm. The correction for solubility (4) in the total of 85 cc. of filtrate was 0.16 gm., a total of 1.05 gm. The calculated yield was 1.18 gm.

*Color Development from the New Compound.*

37.5 mg. of Preparation 13 were dissolved in water, diluted to 500 cc., and compared in a colorimeter with 0.5 N  $K_2Cr_2O_7$  set at 8.0 mm. The reading obtained was 11.0 mm., equivalent to 7.36 mg. of creatinine or 19.6 per cent. 37.2 mg. of the same preparation were dissolved in 10 cc. of water, a mixture of 15 cc. of 1 per cent picric acid and 5 cc. of 10 per cent NaOH was added, and the mixture at once diluted to 500 cc. The reading was 9.1 mm., equivalent to 8.92 mg. of creatinine or 26.9 per cent. This is the calculated amount.

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# AN INHERITANCE STUDY OF THE DISTRIBUTION OF VITAMIN A IN MAIZE.\*

BY SIGFRED M. HAUGE AND JOHN F. TROST.

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The investigations on the correlation between yellow pigmentation of certain foods and their content of vitamin A, have been reviewed adequately (1, 2). Although vitamin A is associated with yellow pigmentation in many plant and animal tissues and in many oils, yet it has been clearly demonstrated that vitamin A may be present in the absence of the yellow pigments and *vice versa*.

It is well known that the yellow dent varieties of corn (maize) contain an adequate supply of vitamin A for normal growth in the albino rat, while the white dent varieties are lacking, or at least highly deficient in this factor. This fact, together with our knowledge that the inheritance of endosperm color in maize is typically Mendelian in nature (3), provides a background for determining the mode of inheritance of this biochemical factor. Because of the rather constant difference in vitamin A content between the yellow varieties of corn and all white ones which have been investigated, it is important to study the association between yellow endosperm and vitamin A content of dent corn in relation to physiological and genetic characteristics.

Such information will be of value to the corn breeder who is crossing yellow and white dent strains of corn for the production of higher yielding strains of high feeding value and with other commercially desirable qualities. It will also be valuable to the

\* Published with the approval of the Director of the Agricultural Experiment Station, Purdue University.

farmers of some sections of the corn belt where certain locally adapted strains of white dent corn consistently give better yields than any of the yellow dent strains which have been introduced up to the present time. Where such differences exist, or when corn is being purchased for live stock feeding, some quantitative measure of vitamin A content of the grain is essential in determining the relative economy of using corn as a sole source of this factor rather than supplementing the ration with materials rich in this constituent.

Although the instances of the simultaneous occurrence of this vitamin and plant carotinoids have been generally accepted to be purely accidental, evidence of their lack of association has not been presented for corn. Comparison of the biological value of the cross white dent  $\times$  yellow dent and its reciprocal, yellow dent  $\times$  white dent, should provide these data. If yellow color is always associated with the higher vitamin A content in the progeny of these crosses, some chemical or physical relationship between the two may be assumed. If a genetic relationship exists under these conditions, it must be a case of close linkage of the genes responsible for these characters.

In the crosses of white dent by yellow dent, it is well known that the first hybrid generation grains appear white-capped or white at the soft starch portion of the crown with distinctly yellow horny endosperm. In the reciprocal cross, yellow by white dent, these first hybrid generation grains appear uniformly light yellow but darker than those of the preceding cross. The  $F_2$  hybrid generation from these seeds consists of dark yellow, medium and light yellow, and pure white grains in the approximate proportion of 1 : 2 : 1. Under such conditions there is a single pair of genetic factors controlling the endosperm color relations between a white dent and yellow dent. The question arises whether or not the vitamin A content is transmitted along with yellow endosperm through the process of crossing and segregation.

Evidence for the presence of additional genetic factors for the development of yellow endosperm has been presented by East (4), Emerson (5), Anderson (6), and Hayes and Brewbaker (7). These dominant factors which lead to the development of pale or light yellow endosperm in comparison with the amber or orange-yellow which is normal to such a variety as Reid Yellow Dent, do not

appear frequently and were not encountered in the material studied.

In this study, the analysis of the vitamin A content of the corn grain in crosses of yellow and white endosperm must be considered from the view-point of a single dominant Y (yellow) and a recessive y (white) factor. Because of the process of double fertilization, there will then be four different genetic combinations possible in the endosperm tissue of the corn grains as follows:

YYY, yellow factors from both parents.

YYy, two yellow factors from female and one white from the male.

yyY, " white " " " " yellow " " "

yyy, white factors from both parents.

An  $F_2$  population of the cross white by yellow endosperm will consist of 25 per cent each of pure yellow and pure white endosperm grains, which may be recognized by inspection and separated from the remaining 50 per cent. The two genetic components of this light yellow class cannot be recognized accurately by inspection, and their separation was not attempted by genetic processes in the study because it was not warranted by the present degree of precision in the quantitative estimation of the vitamin A constituent.

As slight differences in degree of maturity, weathering, and other environmental agencies cause variations in the color of different ears of the same genetic constitution, it is important to reduce such variations in sampling. This was accomplished by having each color class selected in equal quantity from each  $F_2$  ear used. As the different genotypes are distributed at random over the entire ear this procedure should insure a uniform environment for each fraction of the sample.

The material used in this study consisted of single cross hybrids and selected inbred strains of the well known varieties, Johnson County White Dent and Reid Yellow Dent. In the first set of experiments two representative samples of the open pollinated parent strains from which the inbred material and hybrids were derived, were included. The cross 461-5-1-2 (yellow dent)  $\times$  12-14-2-4 (white dent) was made reciprocally and served as foundation stock.  $F_2$  ears were obtained from this material by hand pollination, and equal quantities of each class, yellow, light yellow,

and pure white grains, were selected from each ear to prepare the composites which were dried and ground for the biological analyses. 200 ears were used in 1926 and 100 in 1927 for these composites. The material used in 1926 came from first generation hybrid seed of the cross, yellow dent  $\times$  white dent, while that used the next year came from the reciprocal cross. The purpose of this procedure was to test the possible relation of the vitamin A content to the parent variety characteristics apart from endosperm color.

TABLE I.

*Composition of Rations Used to Test Various Types of Corn for Vitamin A.*

Lot No.....	1	2	3	4	5
	per cent	per cent	per cent	per cent	per cent
White endosperm, parent.....	20				
F <sub>2</sub> white, yy.....		20			
F <sub>2</sub> yellow, Yyy and YYy.....			20		
F <sub>2</sub> yellow, YYY.....				20	
Yellow endosperm, parent.....					20
Casein.....	15	15	15	15	15
Agar-agar.....	2	2	2	2	2
Lard.....	5	5	5	5	5
Yeast.....	3	3	3	3	3
Salt Mixture 185*.....	3	3	3	3	3
Dextrin.....	52	52	52	52	52

All animals were protected against rickets by irradiation with ultra-violet light.

\* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

#### EXPERIMENTAL.

Through the breeding technique previously described, three lots of hybrid corn were secured from the same ears: (1) F<sub>2</sub> yellow, YYY, (2) F<sub>2</sub> yellow, Yyy and YYy, and (3) F<sub>2</sub> white, yyy. With the two parent varieties, (4) Johnson County White Dent and (5) Reid Yellow Dent, five lots of corn were used in these tests.

These experiments were conducted with albino rats selected from our stock which is a pure strain, originally secured from the Wistar Institute. Litter mates were distributed through the various lots of each series, the number and sex being shown by the

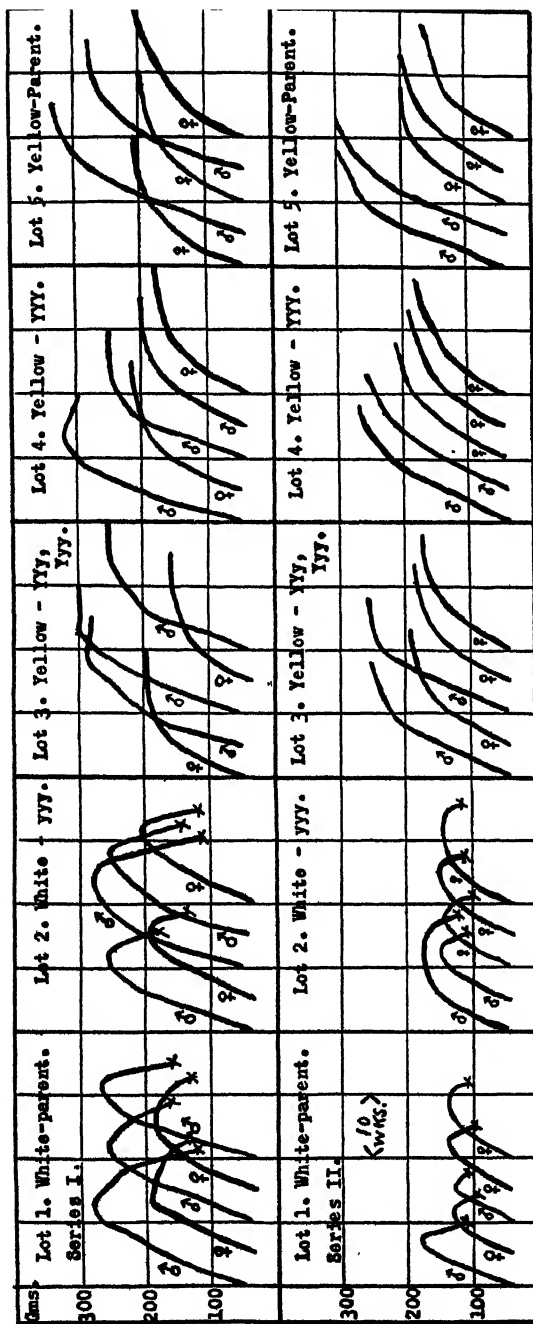


CHART 1. Showing the results of the inheritance study of vitamin A in maize. Series I, comparison of the hybrid kernels of the cross, yellow dent X white dent, with the parent strains. Series II, similar test with the reciprocal cross. Vitamin A was found to be present only in the kernels with yellow endosperms, and lacking in the kernels with white endosperms, even when the latter were grown on the same ears as those with yellow endosperms.



chart. Each rat was placed in an individual cage of a type which prevented access to excreta. The food was given *ad libitum* in the McCollum type feeding cups. The composition of the rations is given in Table I. All animals were protected against rickets by irradiation with ultra-violet light. Weekly records of weights of the animals and of food consumption were kept.

*Series I (Yellow Dent × White Dent).*—The corn used in these tests was produced in 1926. The results of these tests are presented in Series I of Chart 1. In comparing the Lots 2, 3, and 4, it is seen that vitamin A was present only in the kernels possessing the yellow endosperm and was lacking in the kernels of pure white endosperm, even though they were grown on the same ears as those of the yellow endosperm. The results paralleled those of the pure parent strains of white and yellow endosperm.

*Series II (White Dent × Yellow Dent).*—The corn used in this series was produced in 1927. The results of these tests are presented in Series II of Chart 1. It is to be noted that although the  $F_2$  corn was produced from the reciprocal cross of that of Series I, the results were similar. The vitamin A maintained its association with only the yellow endosperm.

#### DISCUSSION.

In regard to the association of vitamin A in corn with the yellow pigment, Palmer (8) says: "The relation between the vitamine and color in the case of corn may be a genetic one, in which case it should be possible to transfer the vitamine to white corn." If such a transfer were possible, it would naturally be an important factor in increasing the nutritive value of some types of corn.

The results of these experiments indicate a close physiological association of vitamin A with the yellow endosperm kernel character in dent corn. It has not been possible to obtain any measurable transfer of this factor to grains possessing pure white endosperm, as rats developed ophthalmia on such corn in all cases here reported. In other tests, not reported, where rats were fed white corn segregated from the cross of yellow dent × white dent, they developed ophthalmia even when the white corn constituted 50 per cent of the ration. In these materials vitamin A was transmitted exclusively with yellow endosperm through the process of crossing and segregation.

Identical results were obtained from yellow dent crossed by white dent and likewise from the reciprocal cross. These facts indicate that any genetic relationship between these two characters must be one of very close linkage.

In these experiments, the ophthalmic reaction was used as the criterion for the presence or absence of vitamin A. All the rats on the rations containing white corn succumbed to ophthalmia, while none of those on the rations containing corn with yellow endosperm developed any symptoms of this disease. Previous to the appearance of ophthalmia, all of the rats made good growth.

At the completion of Series I, it was recognized that the relatively long period before the incidence of ophthalmia was probably due to a heavy storage of this vitamin in the animals, since they were from stock which had received a vitamin-rich ration, fortified with cod liver oil. It, therefore, seemed desirable to use rats in the next series which would have a lower storage of vitamin A. This was accomplished by placing a group of rats on a lower vitamin A diet, omitting the cod liver oil, and protecting the animals against rickets by irradiation. The rats used in Series II were selected from litters of this group. The difference in vitamin A storage of the two groups of animals used in Series I and II, undoubtedly accounts for the more rapid onset of ophthalmia in Lots 1 and 2 in Series II, which is well illustrated by the difference in the graphs of the two series.

At the 20 per cent levels of corn used in these rations, it was not possible to detect any differences in vitamin A content between the segregating genotype homozygous for yellow endosperm and the yellow class heterozygous for this kernel character. These results indicate that hybrid strains of dent corn, produced by crossing white and yellow dent corn, will possess an abundant supply of vitamin A.

#### SUMMARY.

1. A study was made of the association between the vitamin A content and the inheritance of the yellow endosperm kernel character in a cross of Reid Yellow Dent and Johnson County White Dent corn.

2. The vitamin A was transmitted exclusively with yellow endosperm through the process of crossing and segregation.

3. It has not been possible to obtain any measurable transfer

of vitamin A to white endosperm grains selected from the  $F_2$  segregating ears.

4. There is a close physiological association between vitamin A and yellow endosperm kernel character in dent corn. Any genetic relationship must be one of close linkage between these two factors.

5. At the level of corn used in the rations of these experiments, the three classes possessing yellow endosperm, heterozygous  $F_2$ , homozygous  $F_2$ , and homozygous parent, were equally effective in preventing ophthalmia.

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# ANIMAL CALORIMETRY.

## THIRTY-SEVENTH PAPER.

### THE SPECIFIC DYNAMIC ACTION OF GLYCINE GIVEN ORALLY AND INTRAVENOUSLY TO NORMAL AND TO ADRENALECTOMIZED DOGS.

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#### INTRODUCTION.

In a publication of 1922 Lusk (1) says: "The specific dynamic action of protein consists in a specific chemical stimulus of cellular protoplasm, which is independent of the oxidation of the material through which the stimulus is applied. It may be termed the *metabolism of amino-acid stimulation*."

Wilhelmj and Bollman (2) have stated that the specific dynamic action of glycine amounted to more than 3 times the physiological heat value of deaminized glycine and nearly  $1\frac{1}{2}$  times the physiological heat value of the total amount of glycine given. The heat appearing as specific dynamic action after alanine was given was 121 per cent of the heat value of the amount of alanine deaminized. When these authors express the specific dynamic action of the

amino acid as calories for each millimol deaminized they find that the specific dynamic action of phenylalanine is twice that of alanine. In conclusion they emphasize that they do not believe, nor do they wish to imply, that specific dynamic action is always necessarily associated with deamination of the amino acid.

These investigations by Wilhelmj and Bollman seem to tally well with Lusk's view quoted above on the specific dynamic action of amino acids as dependent upon a stimulus which augments metabolism in the organism. As to how such a stimulus to increased metabolism can come about, nothing is as yet known with certainty.

In experiments on rabbits Nord (3) found that the hyperglycemia arising after injection of glycine does not occur if the adrenals have been removed. Nord (4) also found that injection of glycine into rabbits brings with it characteristic changes in the chromaffin tissue of the adrenals, indicating an increased secretion of epinephrine there. These investigations support the assumption that the injection of glycine into rabbits—subcutaneously or intravenously—results in an increased production of epinephrine.

As epinephrine has a decided capacity for augmenting metabolism, it may naturally be asked whether the adrenals have something to do with the production of the specific dynamic action of amino acids. To study this problem we have carried out investigations on dogs in order to discover whether the specific dynamic action of glycine still exists after removal of both adrenals, and whether, if such be the case, this effect is as great as in normal animals. As the experiments by Nord mentioned above were carried out by subcutaneous or intravenous administration of the amino acid, we have in our experiments supplied the amino acid not only *via* the stomach—the classical mode of procedure in experiments concerning specific dynamic action—but also by intravenous injection. That the last mentioned method of applying the amino acid would be useful for the study of the specific dynamic action was likely on the strength of the following data from the literature.

Wolf and Hele (5) obtained considerable specific dynamic action with rapid onset after intravenous injection of glycine in decerebrate dogs. Aub, Everett, and Fine (6) obtained similar results in decerebrate cats. Krzywanek (7) obtained a specific

TABLE I.  
*Experiments on Normal Dogs.*

Dog No.	Date.	Experiment No.	Duration of experiment.	a.q.	Calories per hr.		Increase of indirect calories over basal.		
					Total calou- lated.	Total calou- lated.	cal.	%	
51, weight 8.7-9.5 kg.	1927	Dec. 16	3 hrs.	0.96	18.55	18.42		Basal metabolism.	
		" 20	3 " 1 min.	0.83	16.68	18.53		" "	
		" 22	5 " 4 "	0.80	17.49	16.32		" "	
	1928	" 30	7 " 59.5 "	0.80	18.67	18.82		" "	
		Jan. 3	3 "	0.81	18.45	18.75		" "	
		" 5	10 " 0.5 "	0.80	18.14	17.56		" "	
Average.....				0.83	17.93	17.97			
51	1927	Dec. 21	4 hrs. 0.5 min.	0.82	19.57	19.75	1.64	9.1	
	1928	Jan. 4	4 "	0.85	20.36	20.30	2.43	13.6	
		Feb. 9	3 hrs. 20 min.	0.82	17.43	17.24		Basal metabolism.	
		" 10	2 " 40 "	0.88	17.50	16.38		" "	
		" 14	5 " 40 "	0.86	16.67	15.81		" "	
		" 16	7 " 43 "	0.92	16.92	16.96		" "	
56, weight 11.1-11.3 kg.		" 23	9 " 1 "	0.96	17.53	19.11		" "	
	Average.....				0.89	17.17	16.95		" "
		Feb. 10	3 hrs. 20.5 min.	0.881	21.00	20.35	3.83	22.8	
		" 11	4 " 10 "	0.88	22.12	21.30	4.95	28.8	
		" 15	6 " 20 "	0.92	21.19	20.02	4.02	23.4	
		" 24	10 " 2 " 4 "	0.84	20.43	18.29	8.26	19.0	
Average.....				0.88	21.25	19.87	4.08	23.7	
56		Feb. 10	3 hrs. 20.5 min.	0.881	21.00	20.35	3.83	22.8	
		" 11	4 " 10 "	0.88	22.12	21.30	4.95	28.8	
		" 15	6 " 20 "	0.92	21.19	20.02	4.02	23.4	
		" 24	10 " 2 " 4 "	0.84	20.43	18.29	8.26	19.0	
	Average.....				0.88	21.25	19.87	4.08	23.7
					10 gm. glycine orally.	10 " " intravenously.	10 " " "	10 " " "	

dynamic action by intravenous injection of glycine and alanine into normal dogs. In this laboratory Weiss and Rapport (8) in experiments on a dog after 10 gm. of glycine were given orally obtained a 21.4 per cent increase of the basal metabolism, subcutaneously one of 15.0 per cent, and intravenously an increase of 18.6 per cent. The work by Wilhelmj and Bollman (2) referred to above—published, however, after the conclusion of our work—also shows that the specific dynamic action of amino acids makes itself felt after intravenous injection in the dog. Contrary to all these investigators Liebeschütz-Plaut and Schadow (9) have not found any specific dynamic action in the anesthetized dog after intravenous injection of glycine or other amino acids.

#### *Experimental Procedure.*

The experiments have been carried out with the calorimeter long employed in this laboratory. As experimental animals we have used bitches which prior to the experiments have been trained to lie quietly in the calorimeter. Any movements are detected by a registering device. In case the animal moves sufficiently to influence the metabolism to any appreciable extent, the results are discarded. For some time before the commencement of the experiments and all the time while they lasted, the normal dogs received daily a standard diet. Every afternoon at 5 they received the same diet that has long been used in this laboratory for dogs of similar size (10): 100 gm. of lean beef heart, 100 gm. of biscuit meal, 20 gm. of lard, and 10 gm. of bone ash. The experiments as a rule were commenced 17 to 18 hours after the meal. However, after double adrenalectomy, the animals in some cases refused to take food, which accounts for the low R.Q. in the basal determinations. While this state of undernutrition may somewhat lower the basal metabolism, it would not affect the response to glycine.

The calculations of the experiments have been done in the way usually practised in this laboratory.

All injections have been carried out with due attention to aseptic precautions.

#### EXPERIMENTAL.

The intention was first to investigate the specific dynamic action following oral and intravenous administration of glycine to the nor-

mal animal, and later to repeat the same experiment upon the same animal after the removal of the adrenals. Those animals, however, on which such preliminary tests were carried out, unfortunately died so soon after the removal of the second adrenal that the time at our disposal did not allow any further attempts of this nature. As these preliminary experiments had occupied so much time, we were obliged to proceed directly with extirpation of the adrenals without having examined the specific dynamic action before the operation.

*A. Comparison between Oral and Intravenous Administration of Glycine to Normal Dogs.*

In Table I will be found a tabulation of the experiments on Dog 51. Six experiments gave an average basal metabolism of 17.93 calories per hour. Intravenous injection of 10 gm. of glycine resulted in an average augmentation of the metabolism by 9.1 per cent; oral administration of the same quantity of glycine gave an increase of 13.6 per cent. In Dog 56 (Table I) five experiments gave an average basal metabolism of 17.17 calories. Intravenous injections of 10 gm. of glycine into this animal resulted in an increase of 28.8, 23.4, and 19.0 per cent respectively or an average of 23.7 per cent, and on oral administration an increase of 22.3 per cent was obtained. In these two animals, therefore, four experiments in all were carried out with intravenous injection of glycine, all of them showing an undoubted specific dynamic action. In Dog 51 somewhat less specific dynamic action was obtained on intravenous injection than on oral administration. Dog 56, on the other hand, reacted with a more marked increase of the metabolism on intravenous injection than on oral administration of glycine. In all three experiments on this animal a somewhat more marked specific dynamic action followed after intravenous injection than was obtained by Weiss and Rapport (8) in similar experiments in this laboratory with the same calorimeter. The average increase in our experiments and in those of Weiss and Rapport (see their Table IX) is: orally 19.1 and intravenously 19.8 per cent. Corresponding figures for the absolute increase were: orally 3.26, intravenously 3.39 calories. These figures are so much alike that the difference between them lies within the limits of variability. It would seem, therefore, as if *intravenous and oral administration of glycine in the dog resulted in equally marked increases of metabolism.*



*B. Experiments on Adrenalectomized Dogs.*

The adrenals were extirpated under ether anesthesia with due attention to usual aseptic precautions. In order to diminish the effect of operative shock the adrenals were removed at two sittings at intervals of about 7 days. The right adrenal, being surgically the more difficult, was removed at the first operation, the left at the second. The metabolic tests were commenced on the following day. As the basal metabolism might conceivably alter from day to day after adrenalectomy, it was determined every morning before the glycine experiment was carried out. The value obtained for the basal metabolism the same morning was thus taken as the starting point for the calculation of the increased effect obtained after administration of glycine on the same day. The animals were in good condition at the time of the experiments. Dog 53 died 3 days after the last experiment. No trace of the adrenal could be found at the autopsy. Dog 55 died a couple of days after the test. No postmortem examination was made. Dog 57 was still in excellent condition 43 days after removal of the second adrenal. There was no doubt whatever that both adrenals had been completely removed and that no accessory adrenals could be observed at the operation in spite of careful search. Nevertheless exploratory laparotomy was performed on the 43rd day for further search for any remnant of the adrenals proper or accessory adrenals. No trace of adrenal tissue could be found. Dr. S. F. Cooper who followed the further fate of the dog gives the following particulars: "I killed the dog on June 5, 80 days after removal of the second adrenal. Careful postmortem examination showed no signs of adrenal tissue. Since nothing suspicious was seen no microscopic sections were made. At the time of death the dog was a little weak and quite emaciated. It had a very good appetite, however, and the indications were that it would have lived indefinitely."

A glance at Table II shows that in all the experiments on the adrenalectomized dogs we obtained an increase of the metabolism after administering 10 gm. of glycine either orally or intravenously. The average increase orally was 10.2 per cent, intravenously 12.3 per cent. Corresponding figures in the normal animals were 19.1 and 19.8 per cent. The figures for the basal metabolism, however, were very much higher in the operated animals of the same size,

TABLE II.  
*Experiments on Adrenalectomized Dogs.*

Dog No.	Date.	Experiment No.	Duration of experiment.	R.Q.	Calories per hr.		Increase of indirect calories over basal.		
					Total calories found.	Total calories found.	cal.	%	
33, weight 14.1-13.7 kg.	1928 Jan. 9								Right adrenal removed.
	" 17	2	2 hrs. 0.5 min.	0.76	24.06				Left "
	" 18	3	2 " 40 "	0.79	27.09		3.03	12.6	Basal metabolism.
	" 18	3	2 " 40 "	0.75	23.35		3.32	14.2	10 gm. glycine orally.
	" 19	4	1 " 20 "	0.77	26.67		1.19	5.2	Basal metabolism.
	" 19	5	2 " 40 "	0.74	22.94				10 gm. glycine intravenously.
	" 20	6	2 " 40 "	0.79	24.13				Basal metabolism.
	" 20	7	2 " 40 "						10 gm. glycine orally.
35, weight 11.9 kg.	" 23								Found dead. Autopsy, no trace of adrenals.
	Jan. 23								Right adrenal removed.
	Feb. 27	9	2 hrs. 40 min.	0.85	23.66	21.68			Left "
	" 28	10	2 " 39.5 "	0.83	26.07	27.84	2.41	10.4	Basal metabolism.
37, weight 14.2-14.0 kg.	" 28								10 gm. glycine intravenously.
	Mar. 1								Right adrenal removed.
	" 7	3	1 hrs. 10 min.	0.77	30.12	31.60			Left "
	" 9	4	2 " 43 "	0.78	34.47	33.58	4.35	14.4	Basal metabolism.
	" 9	4	2 " 43 "	0.69(?)	34.97		3.38	9.7	10 gm. glycine orally.
	" 10	5	1 " 20 "						Exploratory laparotomy, no trace of adrenals.
	" 10	6	2 " 40 "	0.72	38.35				Killed. Autopsy, no trace of adrenals.
	Apr. 19								
	June 5								

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and on this ground the percentage figures in these are not directly comparable with those in the normal animals. It would seem more correct to compare the figures of the absolute increase per hour. These figures in the adrenalectomized animals were on an average: orally 2.99 and intravenously 2.87 calories. These figures are more like those obtained in normal animals, which were 3.26 and 3.39 calories. The differences between the figures for normal and adrenalectomized animals would not seem to be sufficiently great to lie outside the limits of variability, which is

TABLE III.  
*Alcohol Checks.*

Date.	Experiment No.	Duration of experiment.	R.Q.	Calories.	
				Indirect.	Direct.
1887					
Nov. 29	248	5	0.666	25.56	26.70
" 30	249	3	0.669	28.46	28.91
Dec. 1	250	5	0.661	25.12	26.59
1888					
Jan. 6	251	3	0.664	14.28	14.42
Feb. 17	252	4	0.666	38.71	37.47
" 22	254	2	0.667	33.95	35.90
Mar. 13	256	3	0.665	24.48	24.92
Average.....			0.665	27.22	27.85

During Experiment 253 on February 21 leaks on the registering tambour outside the calorimeter and in the rubber tubes in the bed were discovered. The R.Q. of Experiment 255, March 12, was 0.647 and is not included in the averages given above.

rendered more apparent by comparing Table I with Table II. Even should the experiments on the adrenalectomized animals have shown a lower specific dynamic action, this could have been explained as due solely to another circumstance, for Wilhelmj and Bollman (2) emphasize that the specific dynamic action can, as it were, be masked in cases when the basal metabolism shows an abnormally high figure for the size of the animal.

It has been shown by the experiments, therefore, *that even in dogs deprived of their adrenals glycine exerts a specific dynamic action after oral as well as intravenous administration and that this effect,*

*reckoned as an absolute increase in the number of calories, is very nearly as great as in normal dogs. It would seem clear from this, then, that the adrenals do not play any significant part in the production of the specific dynamic action of glycine.*

The basal metabolism of the adrenalectomized Dog 55 was 35 per cent higher than that of the normal Dog 56 which had about the same weight. Adrenalectomized Dog 57, which weighed 14 kilos, had a basal metabolism of 34.5 calories per hour, which contrasts with one of 22 calories per hour usually obtained in a dog of that size. The increase exceeds 50 per cent. This contrasts sharply with the work of Aub, Forman, and Bright (11) who found in cats that adrenalectomy markedly reduced the metabolism.

### *C. Alcohol Checks.*

For the purpose of checking the calorimetric determination in the course of the experiments control tests have been made from time to time by burning an alcohol lamp in the calorimeter and thereby determining the combustion quotient, *etc.* The heat production has been calculated from the CO<sub>2</sub> production. See Table III.

### SUMMARY.

In all four experiments with intravenous injection of 10 gm. of glycine, carried out on normal dogs, a clear specific dynamic action was obtained. The values of this effect average about the same as by oral administration of the same quantity of glycine.

Also in all the experiments on adrenalectomized dogs undoubted specific dynamic action was obtained after 10 gm. of glycine were given orally or intravenously. The average increase per hour of the number of calories was very nearly the same as in normal dogs: 2.99 calories orally and 2.87 intravenously, as against 3.26 and 3.39 respectively in normal animals.

It would seem clear therefore that the adrenals do not play any significant part in the production of the specific dynamic action of glycine.

After adrenalectomy the basal metabolism of the dogs was markedly increased.

We would like to express our thanks to Dr. J. E. Sweet in whose laboratories the adrenalectomies were carried out.

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# DIFFERENCES IN SERUM AND PLASMA CONTENT OF CHOLESTEROL ESTER.

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In conducting cholesterol ester determinations in parallel on the serum and plasma of blood from the same animal, discrepancies have been observed. The cholesterol ester content of serum

TABLE I.

Animal No.	Serum.		Sodium citrate plasma.		Potassium oxalate plasma.		Heparin plasma.	
	Total cholesterol.	Ester cholesterol.	Total cholesterol.	Ester cholesterol.	Total cholesterol.	Ester cholesterol.	Total cholesterol.	Ester cholesterol.
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1279	208	144	206	128				
1566	140	104	139	93				
5284	110	77	108	68				
13	152	87	150	55				
1414	195	119					194	119
1462	188	134					189	135
H 8	177	117			175	93	170	114
J 55	203	134			201	119	198	132
5082	197	151			185	128	185	156

was invariably greater than that of plasma while the total cholesterol values showed no differences. Since sodium citrate, 4 mg. per 1 cc. of blood, was being used as the anticoagulant in the blood from which the plasma was taken, other plasma samples were investigated in which potassium oxalate, 2 mg. per 1 cc. of blood, or heparin, 1 mg. per 5 cc. of blood, were used. The results showing the differences observed are given in Table I. All determinations were done on normal cow blood using Bloor's (1)

method for total cholesterol determinations and Bloor and Knudson's (2) for cholesterol ester determinations. All blood samples from an individual animal were drawn in succession from the jugular vein.

The data presented indicate that plasma, obtained from blood in which either sodium citrate or potassium oxalate have been used as anticoagulants, contains less cholesterol bound as ester than does serum from blood drawn from the same animal and at the same time. When heparin was used as the anticoagulant no difference in cholesterol ester content was observed between serum and heparin plasma. Although the differences found to exist between the cholesterol ester content of serum and either citrate or oxalate plasma are not very great, they are, nevertheless, of importance in that they are a source of potential error. Obviously the cholesterol ester values obtained for serum or heparin plasma more nearly represent the correct values for the non-cellular portion of the blood than do the values obtained for citrate or oxalate plasma since either sodium citrate or potassium oxalate appear to be able to cause a partial hydrolysis of that portion of the serum cholesterol generally determined as ester cholesterol.

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## CHOLESTEROL ESTERASE IN ANIMAL TISSUES.

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### INTRODUCTION.

From time to time evidence has been obtained in this laboratory indicating the presence in animal tissues of some very active cholesterol ester-splitting substance. The first instance of this kind was in the case of a young bull experimentally infected with *Bacillus tuberculosis*. 10 days prior to the death of this animal its total serum cholesterol was 90 mg. per cent and the serum ester cholesterol was 58 mg. per cent. As another cholesterol and cholesterol ester reading was desired, blood was taken from the heart at the time of autopsy, 2 days after the animal's death. At this time the total serum cholesterol was 69 mg. per cent while the serum ester cholesterol was 0. Other similar instances may be cited. A calf dead of an intestinal infection was autopsied 24 hours following death and blood taken from the heart at autopsy. Serum from this blood contained 144 mg. per cent total cholesterol but none was bound as ester. (Normal bovine blood serum usually contains from 50 to 70 per cent of the total cholesterol bound with fatty acid as ester.) A rabbit dying of a broken neck was bled 5 minutes post mortem. Its total serum cholesterol was 31 mg. per cent while the serum ester cholesterol was 0. 2 days earlier its total serum cholesterol had been 38 mg. per cent and the serum ester cholesterol was 23 mg. per cent. The complete disappearance of cholesterol ester from blood serum taken from an animal after death is not always observed but in the instances in which it is not observed a marked diminution is found when compared with the most recent determination made during the life of the animal.



The nature of these findings suggested two possible explanations. The first of these was that saturation of the blood with carbon dioxide following death might cause a splitting of cholesterol ester into free cholesterol and fatty acid. The second was that the hydrolysis was due to enzyme action.

Mueller (1) in autolysis experiments with blood and mixtures of blood and liver was unable to detect any evidence of hydrolysis of cholesterol esters indicating the presence of cholesterol ester-splitting enzymes and he disputed the earlier positive results of Shultz (2) and Cytronberg (3). Porter (4) in working with esterases extracted from various animal tissues obtained evidence indicating that cholesterol esterase was present in at least a few tissues although the degree of activity was slight in most instances where it was found to be present at all. The one exception to this was in a preparation from human skin which showed quite marked cholesterol ester-splitting ability. Nomura (5) using extracts of dog, cow, horse, swine, and rabbit tissues observed an enzyme, capable of splitting synthetic cholesterol oleate, present in liver, pancreas, intestinal and gastric mucosa, spleen, kidney, and muscle but not in blood.

#### EXPERIMENTAL.

The possibility that the splitting of serum cholesterol might be due to the saturation of the blood with carbon dioxide following death was considered first.

Several samples of cow blood were drawn, in duplicate, heparin being used as the anticoagulant. Carbon dioxide was allowed to bubble slowly through one sample from each animal for half an hour. The other samples were allowed to stand at room temperature. All were then centrifuged and total and ester cholesterol determinations made on the sera. The results were entirely negative and no evidence was obtained that carbon dioxide caused any change in the serum cholesterol ester content.

The second possibility, that of enzyme action, was considered in some detail and the results obtained are given in accompanying tables.

The experimental procedure was usually as follows: Guinea pig tissues were, with one exception, used as the source of the esterase. Each tissue under test was taken from a freshly killed animal, weighed, and ground in a mortar, 5 cc. of physiological salt solution per gm. of tissue being gradually added. The mixture was then filtered through paper. The extract prepared

in this way was found to contain no cholesterol. The esterase was tested against the cholesterol ester in normal cow serum. 2 cc. of salt solution tissue extract were added to 3 cc. of cow serum in a test-tube, mixed well, and incubated at 37° for 3 hours, no preservative being used. Control tubes contained 2 cc. of physiological salt solution and 3 cc. of cow serum. One experiment was conducted in which a water suspension of cholesterol oleate was used instead of cow serum. After 3 hours incubation the total cholesterol was determined by the method of Bloor (6) and

TABLE I.

Tissue extract.	Guinea pig tissues and cow serum.				Rabbit tissues and cow serum.		Guinea pig tissues and water suspension of cholesterol oleate.	
	Experiment 1. (Incubated 3 hrs.)		Experiment 2. (Incubated 24 hrs.)		Experiment 3. (Incubated 3 hrs.)		Experiment 4. (Incubated 3 hrs.)	
	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Control.....	175	127	156	79	205	170	66	61
Liver.....	174	84	159	55	198	102	72	46
Kidney.....	177	97	157	42	201	107	70	46
Muscle.....	175	98	156	54	203	117	67	52
Lung.....	177	112	153	36				
Brain.....	175	95	158	37				
Spleen.....	175	110	156	54				
Heart.....	174	114	156	54				
Testicle.....	174	108	154	72				

cholesterol ester by the method of Bloor and Knudson (7). The results obtained are summarized in Table I.

The exact temperature at which the cholesterol ester-splitting activity is destroyed was not determined. Boiling the salt solution tissue extract for 5 minutes completely destroyed its ability to split cholesterol ester. The temperature of inactivation lies somewhere below 67° as heating a salt solution extract of guinea pig liver at 67° for 30 minutes yielded the results given in Table II.

The degree of hydrolysis of serum cholesterol ester attained is influenced by the time that the reaction is allowed to progress and

by the concentration of the salt solution tissue extract as shown by the data in Table III. In this experiment an extract of guinea pig liver and normal cow serum were used. In one series the extract was used undiluted as in previous experiments, while in the other series it was diluted 1:4 with salt solution. Samples in each series were incubated for periods of 1, 3, 6, and 24 hours at 37°. 2 cc. of tissue extract and 3 cc. of cow serum were used as before. The control tube was incubated for 24 hours.

TABLE II.  
*Guinea Pig Liver and Cow Serum. Incubated at 37° for 24 Hours.*

Tissue extract.	Total cholesterol.	Cholesterol ester.
	mg. per cent	mg. per cent
Control.....	167	133
Liver.....	167	97
Liver heated to 67° for 30 min.....	168	132

TABLE III.  
*Guinea Pig Liver and Cow Serum.*

Time of reaction.	Undiluted liver extract.		Liver extract diluted 1:4.	
	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.
hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	165	116	167	119
3	168	107	167	114
6	168	104	167	105
24	167	97	168	99
Control (2 cc. salt solution and 3 cc. cow serum).	167	133		

#### DISCUSSION.

The results obtained are in very close agreement with those reported previously by Nomura and are published confirmatory to those observations.

By incubating salt solution extracts of various animal tissues with blood serum or suspensions of cholesterol oleate a decrease

in the cholesterol ester content of the sera or suspensions could be demonstrated with no accompanying change in the total cholesterol. This reaction appeared to be progressive with time and dependent upon the concentration of the tissue extract. Heating the tissue extract to 67° destroyed its ability to split cholesterol ester. The ester hydrolysis is thus probably due to an enzyme that is universally present in animal tissues. All attempts to make the reaction progress to completion, as it apparently sometimes does in the animal body following death, were unsuccessful.

The fact that cholesterol esterase is present in all tissues argues that it has an important and wide-spread function. In view of the complete absence of knowledge concerning the function of cholesterol itself, surmises with regard to the nature of the rôle that cholesterol esterase plays in the animal organism would be unfruitful at this time.

Free cholesterol and cholesterol ester maintain a very constant relationship in the blood serum of a given animal over a period of time and Mueller (8) has shown that the proportion of free to combined cholesterol in the blood serum is approximately the same as that existing in the chyle during the absorption of either free cholesterol or cholesterol ester from the gastrointestinal tract. The fact that during the life of an animal a ratio between free and combined cholesterol is maintained, while soon after death the cholesterol bound as ester decreases, would indicate that the breakdown in the mechanism controlling this relationship was unequal. That is, cholesterol ester formation ceases while hydrolysis of cholesterol ester by an esterase still continues.

#### SUMMARY.

1. Blood serum obtained from an animal post mortem always contains less cholesterol ester than did serum obtained from the same animal preceding death and often contains none.
2. Passage of carbon dioxide into freshly drawn blood has no effect on the cholesterol ester content of its serum.
3. Cholesterol esterase capable of splitting either cholesterol ester in normal cow serum or cholesterol oleate in water suspension is present in many animal tissues.
4. The continued activity of cholesterol esterase post mortem

with cessation either of cholesterol ester formation or absorption may explain the complete absence or diminution of cholesterol ester in blood serum obtained from an animal after death.

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# THE HYPERCHOLESTEROLEMIA OF FASTING AS INFLUENCED BY THE SEPARATE ADMINISTRATION OF FATS, CARBOHYDRATES, AND PROTEINS.

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## INTRODUCTION.

In a previous publication (1) it was demonstrated that the high serum content of cholesterol, associated with fasting, could be rather promptly lowered by the feeding of a mixed meal to the fasting animal. At the time, the suggestion was made that the rise in serum cholesterol observed during periods of fasting might be related to the increased utilization of body fat taking place at that time and the decrease following feeding might accordingly be due to the prompt action of administered food in sparing body fat.

It seemed proper to infer that if the changes in serum cholesterol during and after fasting were truly related to some special functional connection between cholesterol and fat metabolism this would also be reflected in some difference in the influence of feeding with the separate constituents, fat, carbohydrate, or protein, to the fasting animal. This view has been subjected to the test of experiment.

A number of investigators have maintained, on the basis of indirect experimental evidence, that cholesterol has some function in fat metabolism. The data for or against this assumption may be briefly outlined.

Mayer and Schaeffer (2) found that within an animal species for any organ the proportionate content of fatty acid and cholesterol was quite constant. They termed this proportion of fatty acid to cholesterol the "lipocytic coefficient." Terroine and Weill (3) found that inanition caused wide variations in the lipid index of muscle but had little or no effect on that of the parenchymatous organs. Ingestion of food caused a transitory

change in the lipid index of the liver without exerting any influence on that of other tissues. The constancy of the relationship of fats to cholesterol suggested that they were in a combination characteristic of the tissue.

Terroine (4) observed that the ratio  $\frac{\text{cholesterol}}{\text{total fatty acids}}$  in dog blood was a constant for the same animal at different times and very near a constant for the species. However, he noted (5) that during fasting the constant decreased irregularly. Mayer and Schaeffer (6) found that during absolute fasting the ratio of cholesterol to fatty acids of all tissues examined, increased. Morita (7) confirmed this observation. Hueck and Wachter (8) believed that their experiments indicated that cholesterol had a prominent rôle in intermediate fat metabolism. They observed that cholesterol added to food appeared in the blood stream combined as esters with fatty acids, and also that the artificial enrichment of the diet in cholesterol not only led to a hypercholesterolemia but to an increase in the fatty acid fraction of the blood lipoids. Bloor (9) noted a relationship between fat, lecithin, and cholesterol and also between cholesterol and its esters in normal blood that was constant within narrow limits for the individual and within wider limits for the species. Anything tending to result in a disturbance of this equilibrium was followed by an attempt at restoration either by elimination or by an establishment of the relationship again on a higher or lower level. Cholesterol was found to be the most constant and least easily disturbed of the lipid constituents of normal blood. Bloor has repeatedly suggested that cholesterol has some important function in late fat metabolism (10-12). These suggestions have been based largely on the relationship found to exist between blood fat, lecithin, and cholesterol in normal and pathological human blood. Oser and Karr (13), from data obtained on normal human blood, failed to note a constant cholesterol-lecithin ratio. Terroine, Bonnet, Kopp, and Vechot (14) were unable to demonstrate any constant relationship between the aliphatic acid and sterol content of various fatty seeds or microorganisms naturally rich in fat. They did, however, obtain evidence that, in plants and microorganisms, sterols were formed at the expense of the fats. Artom (15), in a comparative study of the variation of the fatty acids and cholesterol content during artificial circulation or autolysis of the liver of a normal dog, was not able to formulate any hypothesis as to the relation between cholesterol and the fats or their cleavage products.

Experimental evidence concerning the effect of ingestion of fat upon the cholesterol content of the blood is conflicting. Beumer (16) found that during digestive lipemia an increase in serum cholesterol occurred. This increase failed to take place in a dog with a biliary fistula indicating that the increase in serum cholesterol during lipemia probably originated in the bile. Bloor (17) was unable to demonstrate any definite changes in blood cholesterol following fat ingestion. Knudson (18) fed dogs olive oil and found no constant changes in their blood cholesterol content during the resulting lipemia. He did, however, find that an increase in the ester fraction of the blood cholesterol occurred and this increase was most

marked in the corpuscles, which normally are considered to contain no ester cholesterol. Chauffard, Laroche, and Grigaut (19) found that the increase in the cholesterol content of the blood observed by them after a meal containing fat was far beyond the amount of cholesterol ingested with the food. Leites (20) fed dogs olive oil and during the resulting hyperlipemia observed an increase in the blood cholesterol, largely as free cholesterol.

TABLE I.

*Effect on Fasting Hypercholesterolemia of Feeding with Pure Carbohydrate.*

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	mg. per cent	per cent
Rabbit 13	Before fasting.	58	26	45
	After 48 hrs. without food.	105	67	64
	3 hrs. after 8 gm. glucose.	90	52	58
Rabbit 14	Before fasting.	59	24	41
	After 48 hrs. without food.	78	43	55
	3 hrs. after 8 gm. glucose.	61	26	43
Rabbit 8	Before fasting.	54		
	After 48 hrs. without food.	83		
	3 hrs. after 10 gm. cane sugar.	71		
Rabbit 17	Before fasting.	41		
	After 48 hrs. without food.	49		
	3 hrs. after 10 gm. cane sugar.	39		
Swine 390	Before fasting.	194	117	60
	After 67 hrs. without food.	240	160	67
	2 hrs. after 200 gm. glucose.	214	119	56
	6 hrs. after 200 gm. glucose.	201	117	58

## EXPERIMENTAL.

The general experimental plan was to fast animals until a definite increase in serum cholesterol content had been established. The fasting period was 48 hours in the case of the rabbits used and 67 hours in the case of the swine. To terminate the fast the animals were fed with either pure carbohydrates (glucose and cane sugar), proteins (gelatin and casein), or fats (olive oil and butter fat). The rabbits had to be fed by stomach tube but this was not necessary in the case of the swine. Blood samples were taken at the



beginning of the period of fasting, just before feeding at the termination of the fast, and at 3 to 3½ hours following feeding in the case of the rabbits and 2 and 6 hours in the case of the swine. Blood from the rabbits was obtained from the marginal ear vein and that from the swine was drawn from the tail. Bloor's (21) method was used for the total serum cholesterol determinations

TABLE II.

*Effect on Fasting Hypercholesterolemia of Feeding with Pure Protein.*

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	mg. per cent	per cent
Rabbit 12	Before fasting.	59	21	36
	After 48 hrs. without food.	86	45	52
	3 hrs. after 8 gm. gelatin.	87	45	52
Rabbit 15	Before fasting.	70	33	47
	After 48 hrs. without food.	121	63	52
	3 hrs. after 8 gm. gelatin.	124	64	52
Rabbit 66	Before fasting.	27		
	After 48 hrs. without food.	49		
	3 hrs. after 8 gm. gelatin.	54		
Rabbit 67	Before fasting.	36		
	After 48 hrs. without food.	56		
	3 hrs. after 8 gm. gelatin.	66		
Swine 392	Before fasting.	142	65	46
	After 67 hrs. without food.	164	100	61
	2 hrs. after 200 gm. casein.	165	103	62
	6 hrs. after 200 gm. casein.	145	76	52

and Bloor and Knudson's (22) for the cholesterol ester determinations. The results are recorded in Tables I to III.

To test further any demonstrable alteration in serum cholesterol content resulting from or accompanying an increase in fat metabolism, four rabbits were given slightly more than their caloric requirements of olive oil for a period of 2 days. They were bled at the beginning of the experiment and at the end of the 1st and 2nd days. The results obtained are given in Table IV.

## DISCUSSION.

The separate administration of either carbohydrate, protein, or fat to an animal, deprived of food for a sufficient period of time to produce a fasting hypercholesterolemia, resulted in all cases in a decrease in the serum cholesterol and cholesterol ester content. Carbohydrate produced perhaps the most marked and rapid

TABLE III.  
*Effect on Fasting Hypercholesterolemia of Feeding with Pure Fat.*

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	mg. per cent	per cent
Rabbit 18	Before fasting.	67	37	55
	After 48 hrs. without food.	86	60	70
	3½ hrs. after 5 cc. olive oil.	77	44	57
Rabbit 19	Before fasting.	50	20	40
	After 48 hrs. without food.	83	50	60
	3½ hrs. after 5 cc. olive oil.	76	46	61
Rabbit 4	Before fasting.	30		
	After 48 hrs. without food.	56		
	3½ hrs. after 5 cc. butter fat.	47		
Rabbit 62 A	Before fasting.	76		
	After 48 hrs. without food.	111		
	3½ hrs. after 5 cc. butter fat.	100		
Swine 391	Before fasting.	130	51	39
	After 67 hrs. without food.	167	97	58
	2 hrs. after 100 gm. butter fat.	154	91	59
	6 hrs. after 100 gm. butter fat.	146	86	59

decrease, due probably to the fact that it was most available to the animal in the fasting condition. There was a very definite latent period following the feeding of protein during which the serum cholesterol either remained constant or rose slightly but the observation on the swine would indicate that this was no more than transient and perhaps represented the period of time necessary for the conversion of protein into a more readily metabolizable substance. Feeding with fat produced a decrease in serum cholesterol and cholesterol ester that was only slightly less marked,

as to rate and extent, than when carbohydrate had been fed. Thus these experiments, so far as they went, failed to demonstrate that the cholesterolemia of fasting could be ascribed to any disproportion in the type of food material being used by the animal at this time as a source of energy. That is, no evidence was obtained to indicate that the fasting hypercholesterolemia was related to the increased utilization of fat taking place at this time

TABLE IV.

*Effect on Serum Cholesterol Level of Feeding Pure Fat to Rabbits (Olive Oil).*

Time of experiment. hrs.	Manipulation.	Rabbit 6.			Rabbit 16.			Rabbit 58.			Rabbit 62 B.		
		Total serum cholesterol.			Total serum cholesterol.			Total serum cholesterol.			Total serum cholesterol.		
		mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent
0	Bled 8 cc. All food removed from cages.	27	7	26	59	19	32	52	17	33	39	13	33
$\frac{1}{2}$	20 cc. olive oil.												
22	20 cc. olive oil.												
24 $\frac{1}{2}$	Bled 8 cc.	38	10	26	58	21	36	55	18	33	46	10	22
46	20 cc. olive oil.												
48 $\frac{1}{2}$	Bled 8 cc.	42	10	24	56	24	44	51	19	37	42	13	31

since feeding with pure fat resulted in a lowering of the serum cholesterol level in much the same fashion as did feeding with carbohydrate, or protein, or a mixed meal. In like manner it was made quite evident that the hypercholesterolemia of fasting was related in no selective way to the metabolism of either carbohydrate or protein.

In view of the amount of indirect evidence indicating some relationship between cholesterol and fat metabolism another type

of experiment was conducted, the effect of fasting upon the blood serum cholesterol content being borne in mind. Four rabbits were maintained for 48 hours on a diet of fat alone. Three of these animals failed completely to show any significant increase in serum cholesterol content while one showed a moderate rise. Observations on other rabbits, fasted for a period of 48 hours, demonstrated that, with no food at all, a very definite increase occurs. It therefore seems evident that the hypercholesterolemia of fasting is not dependent upon the increase in fat metabolism taking place at this time and thus that cholesterol does not have a function in fat metabolism under the conditions of these experiments. If the increased serum cholesterol content of fasting could possibly be considered as bearing a relationship to the increased metabolism of fat taking place at that time then animals fasted except for their caloric requirement in fat should have been expected to show at least the degree of hypercholesterolemia that rabbits fasted for a corresponding period of time would have shown. Such was not the case for, in fact, maintenance for 48 hours on fat alone resulted in no change in serum cholesterol in any except one rabbit. This one animal showed some increase which, however, might very easily be accounted for, in the light of three negative animals, as being due to failure to utilize the fat fed with a resultant fasting hypercholesterolemia.

#### SUMMARY AND CONCLUSIONS.

1. The separate administration of either fat, carbohydrate, or protein to a fasting animal causes a decrease in the hypercholesterolemia similar to that observed following feeding with a mixed meal.
2. A diet of fat alone for 48 hours causes no significant increase in serum cholesterol.
3. The experimental evidence presented adds no weight to the assumption so often made in the past that cholesterol has a function in fat metabolism.

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# THE EFFECT OF AGE ON THE TOTAL AND COMBINED CHOLESTEROL OF THE BLOOD SERUM.

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## INTRODUCTION.

The fact that the physiological function of cholesterol and its esters has not yet been established makes it highly important to utilize every available opportunity to study its variations in the hope of obtaining evidence, which, when added to what is already known, may yield a clearer and more complete picture as to the actual rôle of cholesterol in the physiology of the animal organism. A study of the changes, in both total and combined cholesterol of the blood serum, as related to age, seemed of importance for two reasons. In the first place, it offered an opportunity to observe its variations under conditions, so far, not thoroughly studied. In the second place, in a great deal of work already in the literature, on variations in blood cholesterol as related to various disease processes or physiological phenomena, the factor of age would have vitiated the results had it been considered. It is, therefore, of importance to determine to what extent age does actually influence the cholesterol and cholesterol ester level of the blood serum.

A number of investigators have recognized that differences in blood cholesterol content occurred in organisms of differing ages or stages of development. Slemons and Curtis (1) observed that fetal blood contained an amount of cholesterol approximately equal to the free cholesterol of the maternal blood and it was present entirely in the uncombined state. Chauffard, Laroche, and Grigaut (2) found that blood from the umbilical vein, obtained from infants immediately after birth, contained very much less cholesterol than the maternal blood. The average for four cases was found by these investigators to be 55 mg. per cent. Banu, Negresco, and Heresco (3) noted that the cholesterol content of infants' serum increased from an

average of 47 mg. per cent at the age of 1 to 2 weeks to 55 mg. per cent at the age of 8 to 10 months, both values being extremely low when compared with similar values for adult serum. György (4) observed that funicular serum contained 54 to 83 mg. per cent cholesterol whereas the maternal serum contained from 220 to 279 mg. per cent. Hellmuth (5) confirmed the observations of previous investigators concerning the low cholesterol content of new born infant blood serum but, contrary to the observation of

TABLE I.

*Calves That Had Not Suckled Dam at Time of First Bleeding.*

Animal No.	Age.	Total serum cholesterol.	Combined serum cholesterol.	
		mg. per cent	mg. per cent	per cent
130, Holstein (female).	1 hr.	22	0	0
	48 hrs.	44	17	38
	8 days.	87	38	43
	21 "	153	69	45
	35 "	113	71	63
	57 "	112	68	61
	71 "	157	123	78
	99 "	141	116	82
	148 "	127	88	69
	199 "	93	69	74
132, Holstein (female.)	1 hr.	27	0	0
	48 hrs.	48	18	38
	8 days.	102	44	43
	21 "	165	90	54
	35 "	126	76	61
	57 "	94	61	65
	71 "	122	99	82
	99 "	130	100	77
	148 "	114	84	73
	199 "	91	66	72

Slemons and Curtis, failed to find the total amount of cholesterol present in the free form. Parhon and Parhon (6) found that in extremely old people (over 70 years of age) a definite hypercholesterolemia existed. Roffo (7) observed that in rats the blood cholesterol increased between the ages of 3 and 5 months. Baker and Carrel (8), working with chicken serum, found that the average cholesterol content of that from 3 months old chickens was 225 mg. per cent while that from 4 to 5 years old chickens was 143 mg. per cent.

## EXPERIMENTAL.

Cattle, guinea pigs, and rabbits were the species selected for the study and the results obtained will be given separately for each species. Bloor's method (9) was used for the total cholesterol determinations, and Bloor and Knudson's method (10) for combined cholesterol determinations. All animals used were on an adequate diet and all were bled within a period of 3 hours following feeding (new born calves and guinea pigs excepted).

TABLE II.  
*Calves That Had Suckled Dam at Time of First Bleeding.*

Animal No.	Age.	Total serum cholesterol.	Combined serum cholesterol.	
		mg. per cent	mg. per cent	per cent
134, Holstein (male).	Between 5 and 6 hrs. old.	33	10	26
135, Holstein (male).	Between 5 and 6 hrs. old.	29	8	28
140, Holstein (female).	Approximately 6 hrs.	46	22	48
	48 hrs.	65	36	55
	6 days.	96	55	57
144, Jersey (female).	Approximately 6 hrs.	34	10	29
	48 hrs.	69	31	45
	6 days.	108	74	69

*Cattle.*

Six calves were used, blood being obtained from the external jugular vein. Two of the calves were available for only 1 day and two others for only 6 days after birth. The remaining two calves were available for a period of approximately 7 months. The results are given in Tables I and II.

*Guinea Pigs.*

Because of the small amount of blood that can be safely drawn from the heart of a single guinea pig, determinations were carried out on pooled samples of blood. From four to eight animals were



thus used for each determination. Animals included in the groups whose ages were less than 1 month had not yet been weaned, but all other animals were on the regular diet of hay, oats, and green clover. Only male animals were used. The results obtained are given in Table III.

### *Rabbits.*

Blood was obtained from the marginal ear vein in the case of the rabbits used. A small litter was chosen for the work because

TABLE III.  
*Guinea Pigs.*

Age.	Total serum cholesterol.	Serum cholesterol ester.		Average weight.
		<i>mg. per cent</i>	<i>per cent</i>	
1 hr.	64.5	Trace.		
5 days.	231.3	124.8	53.9	
20-30 days.	63.3	28.7	45.3	136
1-2 mos.	49.6	19.3	38.9	178
2-3 "	49.8	19.9	39.9	230
3-4 "	41.3	15.0	36.3	235
4-5 "	43.2	13.7	31.7	370
5-6 "	38.1	14.6	38.3	460
6-7 "	33.8	11.9	35.2	476
7-8 "	32.8	13.3	40.5	542
8-9 "	33.3	12.0	36.0	540
9-10 "	36.0	15.5	43.0	660
11-12 "	33.3	11.5	34.5	552
16-17 "	23.8	8.7	36.5	640
18-19 "	22.5	7.6	33.7	760
24-25 "	17.0	5.8	34.1	770
29-30 "	15.0	6.3	42.0	700

the animals reached a size large enough for bleeding at an earlier age. Two females and a male comprised the litter. Weaning was completed at 40 days of age. A separate litter of six animals was used in obtaining data regarding the serum cholesterol content of very young rabbits. These animals had nursed previous to the time the blood sample was obtained. They were so small that it was necessary to sacrifice them in order to obtain sufficient blood. The results are given in Table IV.

## DISCUSSION.

The experimental data presented indicate that in herbivorous animals the changes in serum cholesterol as related to age are of two separate types. The first consists in a marked and rather rapid rise from birth for a relatively short period of time, varying with species, during the early life of the animal. The second con-

TABLE IV.

*Rabbits.*

Animal No.	Age.	Total serum cholesterol.	Serum cholesterol ester.		Weight.
			<i>mg. per cent</i>	<i>per cent</i>	
62 (male).	<i>days</i>		<i>mg. per cent</i>	<i>per cent</i>	<i>gm.</i>
	29	114.1	77.8	68.2	660
	45	96.3	53.4	55.4	1040
	65	54.0	21.3	39.4	1450
	134	42.3	5.8	13.7	2395
	162	34.0	8.3	24.4	2500
	292	20.0	9.3	46.5	2720
63 (female).	29	111.8	91.5	81.8	660
	45	104.8	63.4	60.5	1030
	65	62.6	21.7	34.6	1480
	134	76.6	38.3	50.0	3180
	162	58.6	25.6	43.7	2954
	292	52.6	28.0	53.2	3180
64 (female).	29	113.6	73.1	64.3	610
	45	95.2	41.6	43.7	1000
	65	65.0	19.0	29.2	1480
	134	75.6	19.8	26.2	3295
	162	66.0	19.1	28.9	2954
	292	55.6	26.6	47.8	3400
Pooled sample from litter of six rabbits.	3 hrs.	72.9	24.9	29.2	

sists in a less marked and more gradual decline with advancing age. The nature of the animal material available made it impossible to observe the period of increase more than casually in any but one species of animals, cattle, while the period of decrease was observed in guinea pigs, rabbits, and cattle.

The period of increasing serum cholesterol will be considered

first. Blood serum obtained from calves 1 hour after birth and before any colostrum had been taken contained only free cholesterol and that in relatively small amounts. Blood serum of calves between 5 and 6 hours old already contained more cholesterol than at birth and cholesterol was present in the combined as well as the free state. All animals bled at this time had had colostrum which in itself is quite rich in cholesterol and cholesterol ester, especially the first colostrum (11), and could very easily account for the almost immediate onset of the rise. The increase in both total and combined cholesterol was quite regularly progressive for a period of 3 weeks with the combined cholesterol increasing at a greater rate than the total cholesterol. From 3 weeks to around 10 weeks the serum cholesterol and cholesterol ester fluctuated irregularly but the ratio of combined cholesterol to total cholesterol continued to increase quite regularly. Following this and up to 200 days, the end of the experimental period, the serum cholesterol and cholesterol ester decreased quite regularly and progressively and the ratio of combined to total cholesterol decreased also. The small amount of data obtained on very young rabbits and guinea pigs indicates that these two species behave in a manner similar to cattle in that at birth the serum cholesterol content is low and rises subsequently to reach its highest level sometime under 1 month of age.

The period of declining serum cholesterol in guinea pigs and rabbits began some time under 1 month of age and was progressive and gradual. Guinea pig blood serum decreased in its cholesterol content from an average of 231 mg. per cent at 5 days of age to 15 mg. per cent in animals approximately  $2\frac{1}{2}$  years old. The cholesterol ester content of guinea pig serum decreased during the same period of time although in a less regular fashion. The per cent of total cholesterol existing in the combined form fluctuated irregularly and showed no constant changes.

The decline in serum cholesterol content in the case of rabbits was very regular for the only male animal included. Its serum cholesterol decreased from 114 mg. per cent at 29 days of age to 20 mg. per cent at 292 days of age. That the matter of sex is of some importance in determining variations in serum cholesterol with age is indicated by the results obtained with the two female rabbits. While their serum cholesterol decreased with age it did

so in a rather irregular fashion and failed to reach nearly as low a level as that of their male litter mate. The combined serum cholesterol decreased with age also and the percentage in the combined form tended to diminish up until the last bleeding when it rose again in all three animals.

The observation on rabbits regarding the effect of sex in determining variations in serum cholesterol with age is supported by preliminary unpublished experiments on guinea pigs. In these it was found that female animals showed such irregular fluctuations, unrelated in any way to age, that the use of only male animals was resorted to in later experiments.

Regarding cattle, the information at hand is limited to female animals. Both Calves 130 and 132 showed the period of decreasing serum cholesterol content observed in guinea pigs and rabbits and at the age of 199 days had reached the low levels of 93 and 91 mg. per cent respectively. That irregular fluctuations in serum cholesterol content, not related to age, would have been observed in these animals had it been possible to follow them further can be stated definitely, for, in determining the cholesterol content of large numbers of samples of blood serum from lactating cows never have values below 100 mg. per cent been observed. And in the great majority of instances they were much higher. Hence, at some time after 199 days of age, factors, other than age, would undoubtedly have entered to cause marked fluctuations in the serum cholesterol content. That these factors are rather intimately associated with reproduction and lactation is indicated by the observations of Shope and Gowen (12) on variations in the serum cholesterol of cattle during the "dry" period and during early lactation. From the differences observed to exist between the sexes as regards serum cholesterol variation with age it is strongly indicated that the cholesterol in blood serum serves more functions in female than in male animals.

The significance of the variations in serum cholesterol with age is not clear because of the general haziness of our ideas concerning the possible rôles of this substance in physiology. The data presented are insufficient to indicate whether the early increase in serum cholesterol is of exogenous or endogenous origin or whether the decrease in serum cholesterol observed to take place after the preliminary rise is, conversely, a matter of decreased

endogenous production or exogenous intake or both. Any discussion as to the mechanism or function of the changes observed would be little better than a surmise in the light of the deficiencies in our present knowledge concerning the physiological relationships of cholesterol.

#### SUMMARY.

1. Changes in serum cholesterol and cholesterol ester content as related to age are of two separate types. First, there is a marked and rather rapid increase from birth for a relatively short period of time during the early life of the animal. Secondly, there is a less marked and more gradual decline with advancing age.

2. At birth and before having received colostrum, sera from calves contain cholesterol only in the free form and that in very small quantities. Soon after nursing for the first time cholesterol ester appears in the blood serum and the total cholesterol content of the blood serum begins to increase.

3. Changes with age, in the serum cholesterol content, are more uniform and regular in male than in female animals.

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## FEEDING EXPERIMENTS WITH A DIET LOW IN TYROSINE.

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### INTRODUCTION.

Abderhalden (1) removed tyrosine from casein by crystallization, and fed the residue to an adult dog as the sole source of nitrogen. The animal rapidly lost weight. When this food was supplemented with tyrosine there was a gain in weight. Later Abderhalden (2) on feeding rats an amino acid mixture free from *l*-tyrosine found that the nitrogen balance immediately became negative. Upon addition of tyrosine or phenylalanine to the amino acid mixture the nitrogen balance rose, although equilibrium was not attained. This indicates that either tyrosine or phenylalanine is capable of supplying some carbocyclic compound which is required by the animal.

Totani (3) in similar experiments fed rats diets in which the nitrogen was supplied by hydrolyzed gelatin supplemented by amino acids. The addition of tyrosine to the food, believed to be otherwise complete, caused little or no increase in growth. Totani states that this must be explained in one or two ways,—either phenylalanine is capable of replacing tyrosine or animals have the power of synthesizing the benzene ring. Because of the small amount of phenylalanine in gelatin he believes the latter explanation to be the more probable.

Folin and Ciocalteu's (4) method makes possible more accurate information concerning the tyrosine content of experimental diets than has heretofore been available. Since hair hydrolysate is readily freed of a large portion of its tyrosine by crystallization, and because of its low phenylalanine content (5, 6) it was believed possible to obtain a diet adequate in other respects and yet very low in these two amino acids.

### EXPERIMENTAL.

Twenty albino rats at 28 days of age were divided into two groups of ten rats each, in such a manner that litter mates were

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equally distributed between the two. The animals were fed for 12 weeks on diets that differed only in their tyrosine content. The nitrogen of these experimental diets was supplied by gelatin which had been digested by pancreatin, and acid-hydrolyzed human hair, supplemented by amino acids. The gelatin was prepared by dissolving 1 kiló in 5 liters of solution buffered at pH 7 by phosphates. 10 gm. of commercial pancreatin were added and the mixture kept at 38° for 4 days. The 4th day, more pancreatin was added and the digestion allowed to proceed for 4 more days, then placed in a refrigerator. After remaining in the refrigerator for 2 days, it was concentrated *in vacuo* to a thick paste, dried in a vacuum oven at 60°, and ground. Comparison of the values for total nitrogen (7) and amino nitrogen (8) indicates that about one-fourth of the total nitrogen in the gelatin existed as amino nitrogen at the end of the period of digestion. The hair hydrolysate was prepared by boiling the hair for 4 hours in concentrated hydrochloric acid. The mixture was concentrated *in vacuo* at 50° to a thick rubberlike mass. This was dissolved in water by warming on a water bath at a temperature not exceeding 60°. The solution was cooled in a freezing mixture, adjusted to a pH of 5.4, placed in a refrigerator for 48 hours, and then filtered rapidly by suction. The residue on the filter was washed with small quantities of ice water. All washings that did not give a positive reaction to Millon's reagent were added to the original filtrate. The filtrate was concentrated *in vacuo*, dried in a vacuum oven at 60°, and ground.

Diet I was prepared by mixing gelatin (8.8 per cent), hydrolyzed hair (4.5 per cent), tryptophane (0.2 per cent), histidine (0.2 per cent), cystine (0.1 per cent), tyrosine (0.2 per cent), dried yeast (3.0 per cent), salt mixture (4.0 per cent), butter (15.0 per cent), and dextrin (64.0 per cent). Diet II was prepared by omitting the tyrosine in the mixture and an increase of the digested gelatin content to 9.0 per cent. Due to the high chloride content of the hair hydrolysate the Osborne and Mendel (9) salt mixture was modified by omitting the sodium carbonate and hydrochloric acid. Analysis of the food mixture (10) showed a sodium content of 16 mg. per gm. Other data obtained by analysis of the food constituents, and calculations from these, expressing the concentration in the food mixture as fed, are to be found in Table I.

TABLE I.  
*Analyses of Food Constituents.*

	Amino N (7).		Tyrosine (4).		Total N (4).	
	mg. per gm.	mg. per gm. food mixture	mg. per gm.	mg. per gm. food mixture	mg. per gm.	mg. per gm. food mixture
Hair hydrolysate.....	88.9	4.0	0.22	0.009	95.6	4.3
Hydrolyzed gelatin.....	43.3	3.8	0.48	0.042	162.4	14.3
Yeast.....			7.70			
Vegex.....			0.38			

TABLE II.  
*Summary of Animal Weights and Food Intake Record.*

Rat No.	Initial weight.	Final weight.	Gain in weight.	Average food intake per wk.	Average food intake per 100 gm. rat.
Diet I. Females.					
	gm.	gm.	gm.	gm.	gm.
2	51.9	105.0	53.1	49.2	45.7
4	68.0	98.0	30.0	36.5	41.0
5	58.0	95.3	37.3	40.9	44.7
6	62.3	109.2	46.9	39.5	36.1
8	57.8	104.0	46.2	41.9	40.1
9	61.6	98.0	36.4	44.8	49.8
10	60.1	110.0	49.9	48.6	44.2
Diet II. Females.					
15	56.1	101.0	44.9	42.8	40.2
16	58.6	83.5	24.9	31.2	37.2
17	51.1	96.6	45.5	41.9	44.4
Diet I. Males.					
3	72.3	122.0	49.7	64.8	55.1
7	54.6	124.5	69.9	50.0	41.3
Diet II. Males.					
13	65.4	188.3	122.9	72.3	40.0
14	67.5	195.0	127.5	56.8	30.3
19	62.5	128.5	66.0	59.8	48.7
20	62.0	103.5	41.5	48.3	48.7



## Diets Low in Tyrosine

Because the dried yeast did not appear to supply sufficient vitamin B, 1 mg. of Vegex was fed daily after the 3rd week. Animals 1, 11, 12, and 18 died within this period of vitamin B deficiency. Food intake records were made for the 3 final weeks of feeding. Averages of these weekly food intakes and the average

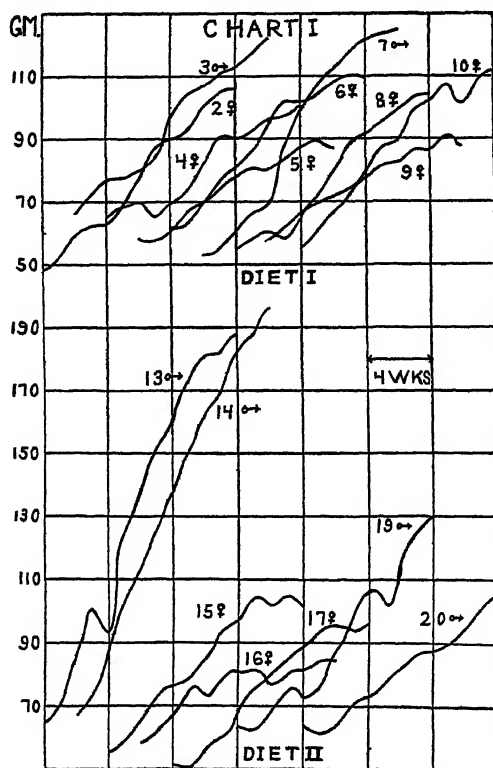


CHART I.

intake per 100 gm. rat are shown in Table II. Growth curves are shown in Chart I.

## DISCUSSION.

The diets as prepared contained 39.7 mg. of tyrosine per 100 gm. of food. Of this 0.9 mg. was derived from the hair hydrolysate,

4.2 mg. from the gelatin, and 34.6 mg. from the yeast. The total nitrogen of the food is slightly in excess of 1.86 per cent. This is the equivalent of 11.55 per cent of protein. Calculated on the basis of average final weights the average daily intake of protein is 0.685 gm. per kilo of rat. The average food intake per week per 100 gm. rat is 44.29 gm. for those animals receiving Diet I, and 41.29 gm. for those receiving Diet II. The average food intake per animal per week is 46.2 gm. for those receiving Diet I and 50.4 gm. for those receiving Diet II. That the food intake is not the factor limiting growth is further shown by Rats 7, 13, and 14. Although these animals made the greatest growth, their food intakes were not the largest. The growth curves show marked variation within each dietary group, and the average final weight of the low tyrosine animals is 11.7 gm. greater than that of the Diet I animals. The variations within the groups, and the difference in final average weights is due to an unequal sex distribution. Thus two of the nine animals receiving Diet I were males, while five of the seven receiving Diet II were males.

#### SUMMARY.

Under the experimental conditions of the present study the growth of rats, for a period of 12 weeks, is independent of the tyrosine content of the food.

The authors wish to thank Dr. H. B. Lewis and Dr. R. C. Huston for their interest and advice during the course of this work.

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## THE DURATION OF THE EFFECT OF ULTRA-VIOLET RADIATION ON CHICKENS.

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The present investigation was undertaken with the object of determining in a quantitative manner the duration of the effect of single doses of the ultra-violet radiation from an artificial source, the quartz mercury lamp. The percentage bone ash, and the calcium and inorganic phosphorus of the blood were selected as criteria to measure the duration of the effect. A search of the literature failed to reveal any report of a quantitative study of the effect of ultra-violet radiations on the chicken, although Hart (1) and his associates state that as little as 3 minutes per week of ultra-violet radiation from a quartz mercury lamp, through glass substitutes, brought about improved calcium assimilation in baby chicks.

### *Experimental Procedure.*

360 14 day old White Leghorn chicks were distributed in six lots of 60 each, so that each reflected the individual weight distribution of the original lot. During the preexperimental period and throughout the experiment the ration supplied consisted of 99 per cent yellow corn plus 1 per cent sodium chloride, and liquid skimmed milk, the two foods being offered separately and *ad libitum* (2).

The ultra-violet radiations were from a Cooper-Hewitt poultry treater lamp, operated on 110 volts A.C., calculated D.C. voltage

\* Cel-O-Glass Industrial Fellow. This investigation was conducted under a fellowship grant from Acetol Products, Inc., New York.

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63 volts, arc wattage 270, placed so that the burner was 3 feet from the surface on which the birds stood. During an exposure they were kept in a circular cage, 3 feet in diameter, whose center coincided with that of the reflector of the lamp.

A sheet of the glass substitute, Cel-O-Glass, was interposed in the case of the lots which received a single exposure in order to prevent possible injurious effects of the shorter wave-lengths. This material does not transmit wave-lengths shorter than 2800 Å. The effectiveness of the radiation through this glass substitute is about one-third of that of the direct rays from a quartz mercury lamp (3). Thus an exposure of 45 minutes through it is equivalent to 15 minutes of the direct rays.

The six lots were given the following treatments: Lot 1, no radiation. Lots 2 to 5, single exposures, on the same day, of 45, 90, 180, and 270 minutes, respectively, through the glass substitute used as a filter. These are equivalent in the effect on bone formation to a direct exposure of approximately one-third of these periods. Lot 6, a daily exposure to 15 minutes direct radiation.

All of the lots were housed in a basement laboratory throughout the experiment. The only daylight which entered the room was from the northeast sky, through window glass.

#### *Analytical Methods.*

Selection for analysis was made by individual weights and the group selected represented the weight distribution of the lot under examination. The ash determinations were made of the individual, dried, alcohol-ether extracted femurs (os femoris) and large wing (humerus) bones (4). Calcium and phosphorus were determined in the serum by the Tisdall (5) modification of the Kramer and Tisdall method and the Briggs (6) modification of the Bell-Doisy method, respectively.

#### *Discussion of Results.*

Seven individuals were killed and analyses made to determine the condition of the whole group a few hours prior to the exposures. For the first 2½ weeks, the analyses of seven individuals from each lot, selected at half-weekly intervals, were used to determine the points on the curves; during the remainder of the

experiment five birds were selected at weekly intervals to determine each point. Analyses were made of individual bones and the results averaged. The blood was pooled for the calcium and phosphorus determinations.

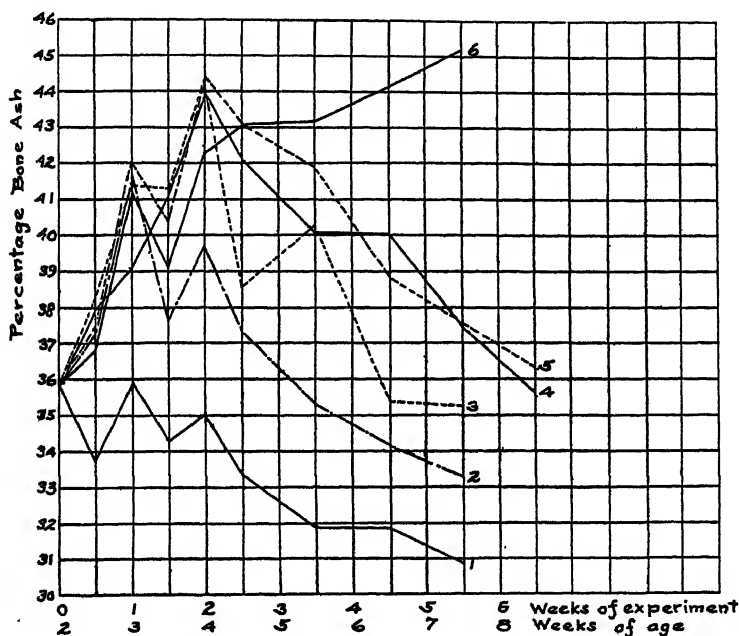


FIG. 1. Percentage of ash of thigh bones. Curve 1, no exposure to ultra-violet light. Curves 2 to 5 represent the effect of single exposures to filtered ultra-violet radiations of 45, 90, 180, and 270 minutes duration, respectively. Curve 6 shows the effect of 15 minutes of direct ultra-violet radiation daily.

That the ration was markedly deficient in vitamin D is amply demonstrated by Curves 1, Figs. 1 to 3, in the case of both the bone ash and blood calcium and phosphorus.

The sharp rise in the femur ash curves, Fig. 1, of the irradiated lots during the 1st week might be due to the fact that the ash percentage, 35.8 per cent, is lower than that observed at 2 weeks of age in an earlier experiment (4), in which the same technique was used, except that daily 15 minute radiations were begun at

5 days of age. In the earlier experiment the bone ash was 39.0 per cent at 2 weeks of age and 42.0 per cent at 3 weeks. Hence, in the present experiment, they reached what is probably normal at 3 weeks of age, at the end of the 1st week of experiment, having started from a lower bone ash content, due undoubtedly to a lack of the antirachitic factor during the first 2 weeks of life.

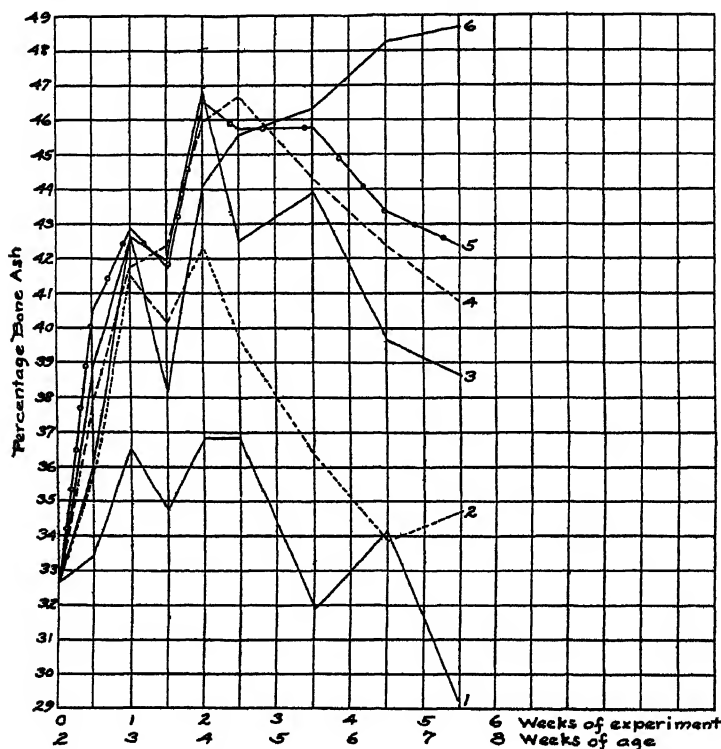


FIG. 2. Percentage of bone ash of wing bones (humerus). Curves 1 to 6, same as for Fig. 1.

The duration of the effect of the 45 minute exposure was for 1 week, after which the bone ash decreased as age, weight, and bone size increased. The rate of decrease is about the same as that of the basal lot. The bone ash of Lots 3 to 5 continued to increase for another week after which the decrease began. In the case of Lots 4 and 5, the decrease in each is at the same rate and

parallels roughly Lot 2 and the basal lot. Curve 3 is somewhat more irregular in its course than the others and its rate of decrease is a little more rapid than any of the other groups. According to the maxima reached, 90, 180, and 270 minutes of single exposure, each causes the same duration of the effect on the mineral

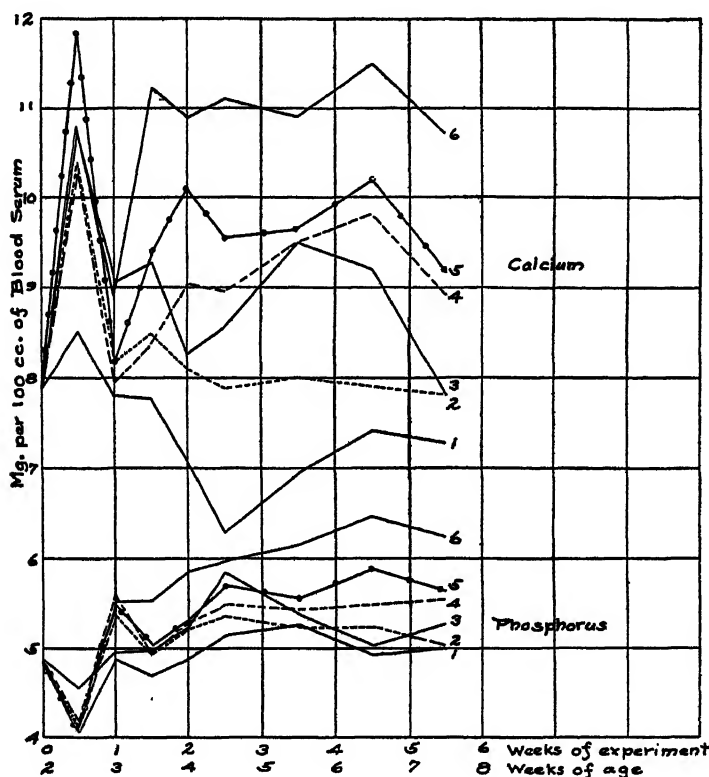


FIG. 3. Calcium and phosphorus of blood serum. Curves 1 to 6, same as for Fig. 1.

deposition in bones, but the rate of decrease of the curves suggests that the 180 and 270 minute exposures may have been slightly more effective than that of 90 minutes.

Results of the ash determinations of wing bones, Fig. 2, portray practically the same picture as do those of the femurs. As in



the case of the femurs, the percentage ash of the wing bones was lower at the beginning of the experiment than that of birds which had received an ample daily dosage of ultra-violet radiation. 40.5 per cent bone ash was observed at 2 weeks of age in an earlier experiment, when an ample daily radiation was given, while in this case it was 32.7 per cent at 2 weeks. At the end of the 1st week of the experiment all of the single exposure lots had reached a value of about 42.0 per cent, which is slightly lower than 43.5 per cent, obtained with ample radiation, and which is probably closer to the normal. At the close of the 2nd week, 4 weeks of age, the curves of the 90, 180, and 270 minute lots had reached a probably normal value for that age, 46 to 47 per cent, and the maximum for these lots. It is of interest that although the initial bone ash percentage of the wing bones is lower than that of the femurs, after the 1st half week, and in some cases at the 1st half week, the percentages are, almost without exception, higher throughout the experiment than those of the femurs. This has been observed in several earlier experiments.

On the same basal ration used in this experiment, but with 1.5 per cent of the corn replaced by Mead's cod liver oil, values of 10.5 mg. of calcium and 6.7 mg. of phosphorus per 100 cc. of serum were obtained at 2 weeks of age. Hence, as in the case of the percentage bone ash of the femurs and wing bones, the initial values shown in Fig. 3 are lower than normal, undoubtedly because of a deficiency of the antirachitic factor during the first 2 weeks of life. The response to the 270 minute exposure is more outstanding in the case of the calcium values than that of those of shorter duration and than the effect on the bone ash. The calcium level of the blood, on the ration used, is probably a more flexible quantity. Although the blood picture, Fig. 3, does not portray the duration of the effect of a single exposure as do the bone ash values, the levels assumed by the curves are, in most instances, of the order of the duration of the exposures.

The sharp increase in the calcium value of each of the radiated lots was followed by a marked drop after which there was another rise to a fairly constant level for several weeks, the curve of Lot 3 showing the greatest fluctuation. Likewise, the curves of the phosphorus values tended toward a constant level as the experiment progressed.

During the first 2 weeks of the experiment, when the fluctuations of the curves were most marked, those of the calcium values were in general opposed to those of phosphorus and the bone ash. The data do not permit a definite interpretation of the relation of these fluctuations to each other, but the sharp rise in the calcium curves and the coincidental drop in those of the phosphorus take place during the time that the mineral content of the bones is increasing rapidly, suggesting an association with the process of bone formation.

Lot 6, which received 15 minutes per day of the direct radiation, was used in order to compare the effects of the various single exposures with the usual procedure of daily irradiation. The response during the 1st week of the experiment was the same as that of the single exposure lots, but the bone ash values are slightly less than those of the 90, 180, and 270 minute lots until the end of  $2\frac{1}{2}$  weeks of experiment, after which the bone ash of the daily exposure lot continues to increase. During the first  $3\frac{1}{2}$  weeks of the experiment the fluctuations of the daily exposure lot, and during the first  $2\frac{1}{2}$  weeks those of the basal lot, parallel those of the single exposure groups. Hence, environmental factors common to all six lots may be in part the cause of these fluctuations.

The observations made in this study suggest the possibility of the use of single exposures at intervals of several days, rather than daily exposures in the practical applications of ultra-violet radiations.

#### SUMMARY.

1. The bone ash percentages demonstrate that the duration of the effect on bone formation of a single exposure of 45 minutes to the filtered radiation (equivalent to 15 minutes of the direct) from a quartz mercury lamp at a distance of 3 feet is 1 week and that for single exposures of 90, 180, and 270 minutes it is 2 weeks.

2. The amount of calcium and inorganic phosphorus of the blood varies directly with the length of the exposure to ultra-violet radiations.

3. The values of the calcium and inorganic phosphorus of the blood fluctuate with relation to the curves of the percentage

bone ash in a manner that suggests a relationship to the process of mineral deposition in the bones.

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## INACTIVATION OF INVERTASE AND RAFFINASE BY HEAT.\*

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The present authors have had occasion partially to inactivate some preparations of yeast invertase by heat. Upon comparison of the activity of the preparations in the hydrolysis of sucrose and the sucrose part of raffinose, before and after the partial inactivation, it was found, in the case of some of the preparations, that there was a greater loss in activity when the latter was calculated from rate of hydrolysis of raffinose than when calculated from the rate of hydrolysis of sucrose. For example the preparation, by means of which the data given in Table I were obtained, suffered a loss of 58 per cent in activity with respect to the hydrolysis of sucrose, while based on the change in activity towards raffinose, the loss amounted to 75 per cent. Several other preparations of yeast invertase showed similar behavior, although in some cases the decrease in activity was the same with respect to both sugars. Why the loss in activity of the different enzyme preparations due to heating was different with respect to the two sugars in some cases and not in others, the present results do not show.

### *Experimental Procedure.*

The procedure followed in obtaining the results given in Table I was as follows: A top yeast (Race-Gebrüder Mayer, *Saccharomyces cerevisiæ*) was grown in molasses, filtered, and to the solid mass of yeast thus obtained, toluene was added, and then the mixture was allowed to stand in a glass bottle and autolyze for about a year at room temperature. The autolyzed material was filtered and the filtrate dialyzed in collodion bags against running

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tap water for 10 days. The dialysate thus obtained was clarified by filtration through infusorial earth. To about 200 cc. of the clear enzyme solution was added sufficient 0.02 M sodium citrate

TABLE I.

Experiment I. 150 cc. 5 per cent sucrose solution, containing 25 cc. unheated enzyme Solution A and 0.02 M sodium citrate buffer. pH 5.0. Samples 25 cc. Temperature of hydrolysis and polariscope readings 25°.

Time, min.	31	75	132	182	30	90	153	190
Change in rotation.	0.11°	0.24°	0.42°	0.59°	0.10°	0.31°	0.52°	0.62°
Initial velocity.	0.003306°; corrected velocity, 0.011009° per min.							

Experiment II. 150 cc. 5 per cent sucrose solution, containing 25 cc. heated enzyme Solution B. Other conditions as in Experiment I.

Time, min.	36	111	189	229	44	113	171	190
Change in rotation.	0.15°	0.49°	0.84°	1.03°	0.20°	0.49°	0.81°	0.85°
Initial velocity.	0.004622° per min.							
Loss in activity.	(From Experiments I and II) 58 per cent.							

Experiment III. 150 cc. 2.05 per cent hydrated raffinose solution, containing 50 cc. unheated enzyme Solution A. Other conditions as in Experiment I.

Time, min.	80	135	196	271	70	124	154	181
Change in rotation.	0.125°	0.21°	0.325°	0.375°	0.105°	0.18°	0.23°	0.28°
Initial velocity.	0.001525°; corrected velocity 0.005078° per min.							

Experiment IV. 150 cc. 2.05 per cent hydrated raffinose solution, containing 50 cc. heated enzyme Solution B. Other conditions as in Experiment I.

Time, min.	93	168	230	282	58	107	167	243
Change in rotation.	-0.11°	0.20°	0.29°	0.33°	0.08°	0.14°	0.21°	0.30°
Initial velocity.	0.001250° per min.							
Loss in activity.	(From Experiments III and IV) 75.4 per cent.							

The initial velocity in Experiments I and III was multiplied by 3.33 in order to correct for the more dilute concentration of the enzyme in the unheated Solution A as compared to Solution B.

solution to give an acidity of pH 5.6. The enzyme solution was then divided into two equal portions, and one of these was placed in a thermostat and heated at 60° for an hour.

*Enzyme Solution A.*—10 cc. of the unheated portion were di-

luted to 1 liter by the addition of 0.02 M sodium citrate solution. This enzyme solution possessed a pH value of 5.0.

*Enzyme Solution B.*—30 cc. of the above heated portion were diluted to 900 cc. with the same 0.02 M sodium citrate solution as was used for Solution A (pH 5.0). Solution B was made more concentrated than Solution A in order to make the slower rates of hydrolysis more convenient to read. Since the rate of hydrolysis is proportional to the concentration of the enzyme, this difference in dilution was taken into account in the corrected velocity values given in Table I.

*Determination of Loss in Activity by Comparing Rates of Hydrolysis of Sucrose by Heated and Unheated Enzyme.*—25 cc. portions of the enzyme Solutions A and B were added respectively to each of two 125 cc. sucrose solutions. The sucrose solutions had been prepared previously by dissolving 7.5 gm. of sucrose in sufficient 0.02 M sodium citrate solution to give a final volume of 125 cc. (same citrate as used in making up Solutions A and B). All the solutions, both enzyme and sugar, were kept in the thermostat at 25°, the temperature at which the hydrolyses were run, for some time before the enzyme was added to the sugar solutions.

The concentration of the enzyme in the sugar solutions undergoing hydrolysis was so small that the rate of hydrolysis was very slow, thereby permitting the taking of a sample for the initial reading a minute or two after the reaction had started. 25 cc. samples were taken from time to time and the hydrolysis stopped in each sample by the addition of a drop of saturated sodium hydroxide solution. The readings were made in 200 mm. polariscope tubes at 25° temperature, with a mercury quartz lamp as the source of light.

The velocities of hydrolysis given in Table I are given in degrees change in rotation per minute. These values were obtained as follows: The polariscopic reading of each sample taken from the solution undergoing hydrolysis was subtracted from the reading of the first sample taken at the beginning of the hydrolysis. The change in rotation thus obtained was plotted as ordinates against the corresponding time of hydrolysis. By drawing a tangent to this curve at its zero point, the value of the initial velocity, the velocity at the beginning of the hydrolysis, when very little invert sugar was present to influence the reaction, was obtained in

degrees change in rotation over minutes, or degrees change per minute.

*Determination of Loss in Activity by Comparing Rates of Hydrolysis of Raffinose by Heated and Unheated Enzyme.*—50 cc. portions of a raffinose solution were added to 100 cc. portions of each of the two enzyme Solutions A and B. The raffinose solution was prepared by dissolving 6.766 gm. of hydrated raffinose in 110 cc. of 0.02 M sodium citrate solution. The rest of the procedure was the same as that followed in the case of the sucrose hydrolyses.

The hydrated raffinose used gave  $[\alpha]_{488}^{25} = +122.4^{\circ}$ . The enzyme solution contained no melibiase. 100 cc. of a solution containing 2 gm. of hydrated raffinose, 25 cc. of the original undiluted enzyme solution (the same solution as used in preparing Solution A), and 0.02 M sodium citrate buffer, were permitted to stand for several days until constant rotation was attained. The rotation of the raffinose solution before hydrolysis was  $+4.94^{\circ}$  and this dropped to  $+2.52^{\circ}$ . Calculated change in rotation, if the raffinose had been hydrolyzed to melibiose and fructose, is  $2.54^{\circ}$ , and if to galactose, glucose, and fructose then it is  $+0.48^{\circ}$ . (Mercury light was used.)

The fact that there is a difference in the degree of inactivation of the invertase and raffinose when a yeast enzyme solution is heated, may have some bearing upon the question whether or not these two enzymes in yeast are identical. Several papers have appeared recently dealing with this question, and the claim is now made by Willstätter and Kuhn (1) and by Josephson (2) that the two enzymes are the same. The question is also discussed in some of the more recent books dealing with enzyme chemistry (3).

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## EXPERIMENTS ON THE NUTRITIVE PROPERTIES OF GELATIN.

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Numerous attempts have been made in the past to supplement gelatin with amino acids in such a fashion as to render it capable of maintaining body weight or of preserving nitrogen equilibrium. Most of these efforts have met with complete failure. A few, notably those of Kauffmann (1905) and Abderhalden (1912), appear at first glance to have been somewhat more successful. An examination of the data of these authors, however, reveals the fact that the periods upon gelatin diets during which nitrogen equilibrium was approximated were of exceedingly short duration. Even if it be granted that gelatin with suitable amino acid additions suffices for the maintenance of nitrogen equilibrium in animals for periods of 5 to 10 days, one would scarcely be justified in assuming on the basis of such evidence that the same would be true for longer periods of time. Furthermore, most of the studies of the nutritive properties of gelatin were made before the recognition of the importance of the vitamins as dietary factors.

Since the development of the newer aspects of nutrition during the past two decades very few experiments upon gelatin have been reported. In their classical studies with isolated proteins, Osborne and Mendel (1912-13) showed that this substance is quite incapable of serving as the sole source of nitrogen. Rats placed upon diets of gelatin, starch, lard, and "protein-free milk" rapidly declined in weight, but when half of the gelatin was replaced by casein or gliadin, recovery with maintenance or growth ensued. Osborne and Mendel did not attempt to correct the deficiencies of gelatin by the addition of individual amino acids, but they point out (p. 242) that the "failure to be maintained . . . is not to be ascribed to a failure in utilization; for nitrogen determinations made on the feces showed that the utilization of the protein was good."

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\* Most of the experimental data in this paper are taken from a thesis submitted by Richard W. Jackson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.



Later Totani (1916) attempted to render gelatin adequate as a source of nitrogen for rats by supplementing the diets with purified amino acids. He reports that the addition of tryptophane alone, or of tryptophane with tyrosine, is without influence upon the quality of the nitrogen. Animals upon gelatin diets, either with or without the supplementing amino acids, suffer 40 per cent losses in body weight in the course of a single month. The author states that hydrolyzed gelatin proved to be a much better source of nitrogen inasmuch as rats receiving this material experienced losses of only 18 per cent in a month. Furthermore, the inclusion of tryptophane in the hydrolyzed gelatin diet led to approximate maintenance. The further addition of tyrosine did not lead to better growth than was secured with tryptophane alone. When tryptophane, tyrosine, cystine; and histidine were incorporated in the ration the results are said to have been "somewhat more satisfactory." The author concludes (p. 397) that, "In the case of the rat, the nutritive efficiency of gelatin is greatly increased by previous hydrolysis. Gelatin when fed intact appears to be badly digested and absorbed." This statement is not in accord with the experience of Osborne and Mendel as outlined above. It is also contrary to the findings of numerous investigators who have studied the utilization of gelatin in animals other than the rat. Thus Reilly, Nolan, and Lusk (1898) report that in diabetic dogs sugar is formed from gelatin in about the same amount as from other proteins. McCollum (1911-12) and Robison (1922), in experiments upon the pig and man respectively, have shown that the ingestion of relatively large quantities of gelatin increases the fecal nitrogen very slightly over the starvation output. Even in the rat, McCollum, Simmonds, and Pitz (1917) have demonstrated that gelatin is capable of supplementing the proteins of rolled oats in a remarkable fashion. These and other experiments reported in the literature would seem to indicate quite conclusively that whatever may be the nutritive deficiencies of gelatin they are not associated with an incapacity of the alimentary enzymes to accomplish its digestion.

In connection with certain investigations in this laboratory we have undertaken a further study of the inadequacies of gelatin as a source of nitrogen for maintenance and growth. On the basis of the most reliable analyses of gelatin it would seem that the amino acid deficiencies are quite evident and could be easily remedied. That the problem is not so simple as it appears will be brought out in the following pages.

Early in our work we observed that the addition of the three conspicuously absent amino acids, cystine, tryptophane, and tyrosine, did not give uniformly the growth desired. It was decided, therefore, to extend the scope of the investigation so as to include not only the amino acid deficiencies, but any other factors

which conceivably might alter the nutritive value of the protein. The following are some of the questions which suggested themselves:

1. Since the very careful and complete analysis of gelatin made by Dakin (1920) reveals the absence of valine, hydroxyglutamic acid, and isoleucine, it seemed not improbable that one or more of these might be limiting factors.

2. Inasmuch as certain other components of gelatin are present in smaller amounts than occur in casein, it appeared necessary to determine the influence upon growth of suitably increasing their intake, either by adding the pure amino acids themselves, or by raising the level of the gelatin in the diet.

3. The pronounced disproportion in the distribution of amino acids in gelatin as compared with casein, particularly as regards the high content of glycine and alanine in the former, suggested that an *improper balance* between the amino acids might exert an influence upon nutrition. Evidence for or against this conception might be secured by incorporating in an otherwise satisfactory diet amounts of glycine and alanine comparable to those present in gelatin.

4. It has been the experience of many that the ingestion by the human subject of large quantities of gelatin is exceedingly difficult. Indeed, depression and nausea almost invariably result. In commenting upon this observation Howell (1925, p. 288) says, "A fact of this kind indicates that the sensory apparatus of the appetite is influenced in some specific way by the metabolism of this particular material." In order to determine whether the peculiar physical or chemical nature of gelatin exerts a deleterious effect upon the organism, we have followed the growth of animals which received a third of their diet in the form of this protein in addition to an abundance of nitrogen of good quality.

5. If gelatin contains amino acid linkages incapable of cleavage by the digestive enzymes, complete hydrolysis of the material before its incorporation in the diet should improve the quality of the nitrogen, as is reported to be the case by Totani (1916). A reinvestigation of this point seemed necessary.

## EXPERIMENTAL.

White rats served throughout as the experimental animals. Invariably they were placed upon a casein diet for brief periods at the beginning of the experiments in order to test their growth propensities. In view of experience gained in the course of the

TABLE I.  
*Composition of Diets.*

Diet.....	A.	B.	C.	D and E.*	F.	G.	H and I.*
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Casein.....	18.0	18.0	18.0				
Gelatin.....			35.0	35.00	30.00	25.00	15.00
Dextrin.....	34.0	22.0		15.30	16.30	20.90	24.00
Sucrose.....	15.0	15.0	14.0	15.00	15.00	15.00	15.00
Salt mixture.....	4.0	4.0	4.0	4.00	4.00	4.00	4.00
Agar.....	2.0	2.0	2.0	2.00	2.00	2.00	2.00
Cod liver oil.....	5.0	5.0	5.0	5.00	5.00	5.00	5.00
Lard.....	22.0	22.0	22.0	22.00	22.00	22.00	22.00
Tyrosine (l).....				0.90	0.90	0.90	0.90
Cystine (l).....				0.50	0.50	0.50	0.50
Tryptophane (l).....				0.35	0.35	0.35	0.30
Histidine monochloride (l)....					0.26	0.32	0.40
Valine (dl).....					2.70	2.70	4.00
Phenylalanine (dl).....					0.60	0.80	2.00
Glutamic acid (d).....					2.10	2.40	4.00
Aspartic " (l).....							1.00
Leucine (dl).....							1.33
Isoleucine (dl).....							1.33
Norleucine (dl)....							1.33
Glycine.....		9.0					
Alanine (dl).....		6.0					

\* Diets E and I contained hydrolyzed gelatin but in other respects were exactly like Diets D and H respectively. With the exception of Diets E and I, all of the gelatin diets contained the protein in an unhydrolyzed form.

investigation to the effect that younger animals do not seem to withstand the change to the gelatin diets as satisfactorily as do older ones, we permitted all rats employed in the experiments reported herein to attain weights of at least 100 gm. before being transferred from the casein ration. In the conduct of the experiments and in the care of the animals essentially the methods of

Osborne and Mendel as described by Ferry (1919-20) were followed.

The composition of the diets is shown in Table I. In addition to the articles of food enumerated, vitamin B was furnished to each animal daily in the form of tablets containing 50 mg. of commercial yeast extract or 200 mg. of whole yeast.<sup>1</sup> The gelatin was a special product<sup>2</sup> which in manufacture had not been subjected to the action of bleaching agents. It consisted of white, opaque flakes, which were thoroughly dried *in vacuo*, ground, and passed through a 40 mesh sieve. A 1 per cent solution exhibited a faint Millon test, and a negative Hopkins-Cole reaction. Hydroferrocyanic acid gave a very faint turbidity.

The hydrolyzed gelatin employed in Diets E and I was prepared exactly according to the procedure followed by Dakin (1920) in the analysis of this protein with a recovery of over 90 per cent of the component amino acids. For this purpose, a mixture composed of 250 gm. of gelatin, 300 gm. of concentrated sulfuric acid, and 650 gm. of water was heated to boiling for 20 hours at atmospheric pressure. The resulting solution was autoclaved at 135-140° for 8 hours. After dilution, the sulfuric acid was removed with barium hydroxide, and the filtrate and washings from the barium sulfate were combined and evaporated to dryness. The final material, after grinding, was light brown in color.

The inorganic portion of the rations was supplied in the form of Osborne and Mendel's (1919) salt mixture. Of the amino acids used in supplementing the diets, tyrosine, cystine, tryptophane, histidine monochloride, glutamic acid, and glycine were prepared in this laboratory from proteins. The aspartic acid was the natural levo variety secured from Kahlbaum. The valine, phenylalanine, leucine, isoleucine, norleucine, and alanine were racemic compounds most of which were synthesized in the organic laboratory of this University. Both the natural and racemic acids were recrystallized until they gave theoretical values for total or

<sup>1</sup> The extract was prepared by the Harris Laboratories, Tuckahoe, N. Y., while the whole yeast was secured from the Northwestern Yeast Company, Chicago. Both the extract and the yeast were thoroughly tested as to their growth-promoting properties before being employed in the gelatin experiments.

<sup>2</sup> Secured from the Wilson Laboratories, Chicago.

amino nitrogen. Whenever a racemic acid was used, twice the calculated amount was incorporated in the ration so as to insure the presence of the active enantiomorph in the quantity desired.

In supplementing the gelatin diets we have used the composition of casein as our guide. On the basis of the most reliable figures for casein composition (*cf.* Mendel, 1923, p. 115), and the analysis of gelatin by Dakin (1920), we have calculated the quantity of each known amino acid<sup>3</sup> present in 100 gm. of food containing 18 per cent of casein and 35 per cent of gelatin respectively. These figures are presented in the first two columns of Table II. In the other columns of this table are shown similar data for the supplemented gelatin diets employed in this investigation.

In most of our experiments gelatin was incorporated in the food at a 35 per cent level. This relatively high proportion was adopted instead of the customary 18 per cent because the protein in question contains several amino acids in quantities far in excess of the accepted values for casein. A comparison of the composition of the two proteins indicates that gelatin yields superfluous amounts of glycine, alanine, hydroxyproline, and arginine. Even if we assume that quantities of these amino acids considerably larger than are supposed to be present in casein are required by the organism, we are still confronted with the necessity of feeding almost double the proportion of gelatin as compared with casein in order to insure the presence of the other amino acids in approximately comparable amounts. A diet containing 35 per cent of gelatin seemed, therefore, a reasonable starting point.

*Growth on a Gelatin Diet Supplemented with Three Amino Acids.*—The data presented in Table II make clear that in addition to tyrosine, cystine, and tryptophane, gelatin is wanting also in valine, isoleucine, and hydroxyglutamic acid. Furthermore, on a 35 per cent basis, phenylalanine, glutamic acid, and histidine

<sup>3</sup> During the progress of this investigation methionine, the new sulfur-containing amino acid isolated from casein by Mueller (1923), was identified by Barger and Coyne (1928). For obvious reasons this substance was not taken into consideration in our experiments. Mueller reports its presence in gelatin, but apparently in much smaller quantities than in other proteins. At a later date we expect to determine the relation of methionine to the nutritive properties of gelatin.

are somewhat below the levels present in the 18 per cent casein diet (Diet A) taken as our standard. In our first experiments, we have supplied only tyrosine, cystine, and tryptophane. Thus the resulting gelatin diet (Diet D) was presumably devoid of valine, isoleucine, and hydroxyglutamic acid. There existed also the possibility that it might be somewhat inadequate in respect

TABLE II.

*Amino Acids\* Present in 100 Gm. of the Various Diets as Compared with 35 Per Cent of Unsupplemented Gelatin.*

	35 per cent gelatin.	Diet A.	Diets D and E.	Diet F.	Diet G.	Diets H and I.
	gm.	gm.	gm.	gm.	gm.	gm.
Glycine.....	8.93	0.08	8.93	7.65	6.38	3.83
Alanine.....	3.05	0.33	3.05	2.61	2.18	1.31
Valine.....	0	1.43	0	1.35	1.35	2.00
Leucine.....	2.49	1.75	2.49	2.13	1.78	1.73
Isoleucine.....	0		0	0	0	0.66
Norleucine.....						0.66
Serine.....	0.14	0.09	0.14	0.12	0.10	0.06
Phenylalanine.....	0.49	0.70	0.49	0.72	0.75	1.21
Tyrosine.....	?	0.81	0.90	0.90	0.90	0.90
Proline.....	3.33	1.37	3.33	2.85	2.38	1.43
Hydroxyproline.....	4.94	0.04	4.94	4.23	3.52	2.12
Aspartic acid.....	1.19	0.74	1.19	1.02	0.85	1.51
Glutamic ".....	2.03	3.92	2.03	3.84	3.85	4.87
Hydroxyglutamic acid.....	0	1.89	0	0	0	0
Histidine.....	0.32	0.45	0.32	0.47	0.49	0.46
Arginine.....	2.87	0.69	2.87	2.46	2.05	1.23
Lysine.....	2.07	1.37	2.07	1.77	1.43	0.89
Tryptophane.....	0	0.27	0.35	0.35	0.35	0.30
Cystine.....	?	Present.	0.50	0.50	0.50	0.50

\* The figures are for the *active* amino acids. When racemic acids were employed, only half of each quantity added to a diet is included in the above data.

to phenylalanine, glutamic acid, and histidine. The absence of hydroxyglutamic acid was disregarded in all of our experiments inasmuch as Osborne, Leavenworth, and Nolan (1924) have pointed out that edestin, which does not contain this amino acid, is capable of supporting normal growth.

Of twenty-six rats placed upon Diet D, thirteen died at intervals

varying from 9 to 48 days. The curves representing the body weight changes of two of the animals which failed to survive (Rats 138 and 150) are reproduced in Chart I, and are typical of all except for the length of time which elapsed before death. The curves show profound nutritive failure accompanied by

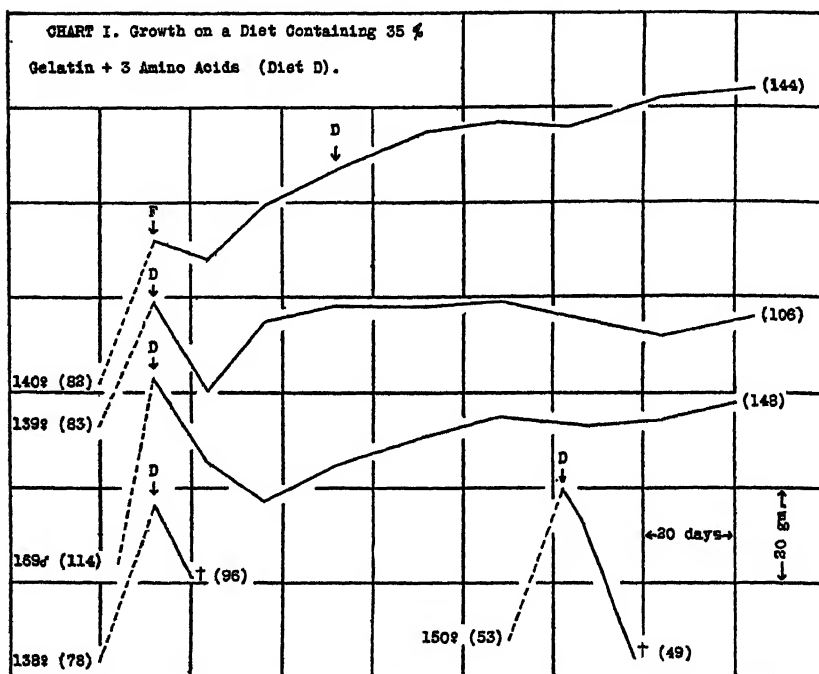


CHART I. The numbers in parentheses signify the initial and final weights of the rats. The broken line portion of each curve represents the growth of the rat during the preliminary period upon Diet A. The solid lines show the growth upon the experimental rations. The arrows denote the points at which the dietary changes were made. The diets are designated by the letters above the arrows.

amazingly rapid losses in weight. In some instances the food consumption dropped almost to zero during the last few days preceding death. At autopsy most of the animals which had died displayed badly deteriorated kidneys. Hemorrhagic lesions were observed in one case, while the exteriors of the kidneys of several

others showed soft, pale, and pitted areas. We are inclined to believe that the renal involvement, rather than the nutritive failure *per se*, was responsible for the early fatal outcome in so many of our experiments. Nor does it seem likely that this can be attributed primarily to the presence of the free amino acids in the ration since numerous experiments in this laboratory (Rose and Huddlestun, 1927) have shown that the incorporation in casein diets of 1.5 per cent of tyrosine, cystine, or tryptophane does not inhibit growth or induce renal failure in rats of 100 gm. weight.<sup>4</sup> Apparently, the effects outlined above are associated with the gelatin factor.

The thirteen animals which survived the transfer to the gelatin ration showed variable but usually very rapid losses in weight immediately after the dietary change, but subsequently improved. We believe that they also experienced kidney injury, but happened to be better adapted to or more successful in adjusting themselves to the adverse conditions than were their fellows who succumbed. The growth curves secured in three long continued experiments are reproduced in Chart I. The typically rapid decreases in weight on the gelatin ration are seen in the curves of Rats 139 and 169. These animals lost 19 and 26 gm. respectively before recovering from the effects of the ration. Subsequently, slow growth followed by practical maintenance ensued. The experiment upon Rat 140 was slightly different from those upon Rats 139 and 169 in that the former animal was transferred from the normal diet to one containing 30 per cent of gelatin supplemented with seven amino acids (Diet F). After it had remained upon this ration for 40 days, Diet D was substituted. This rat showed a smaller decrease followed by a greater increase in weight than either of the others. When the experiment was discontinued at the expiration of 132 days, a net gain of 32 gm. had been made since the transfer from the casein ration.

In Chart II are reproduced growth curves secured in shorter experiments with Diet D. The rats were litter mates. The data are of interest as illustrations of the wide individual differences

<sup>4</sup> Recent unpublished experiments by G. J. Cox of this laboratory have shown that rats weighing 40 to 50 gm. each are rendered nephritic by cystine administration. Under identical conditions, animals of 100 gm. weight manifest no ill effects.



which one encounters in dietary studies involving the use of gelatin. Four of the animals of this litter (curves not shown) died within 2 weeks after the change to Diet D. Two (Rats

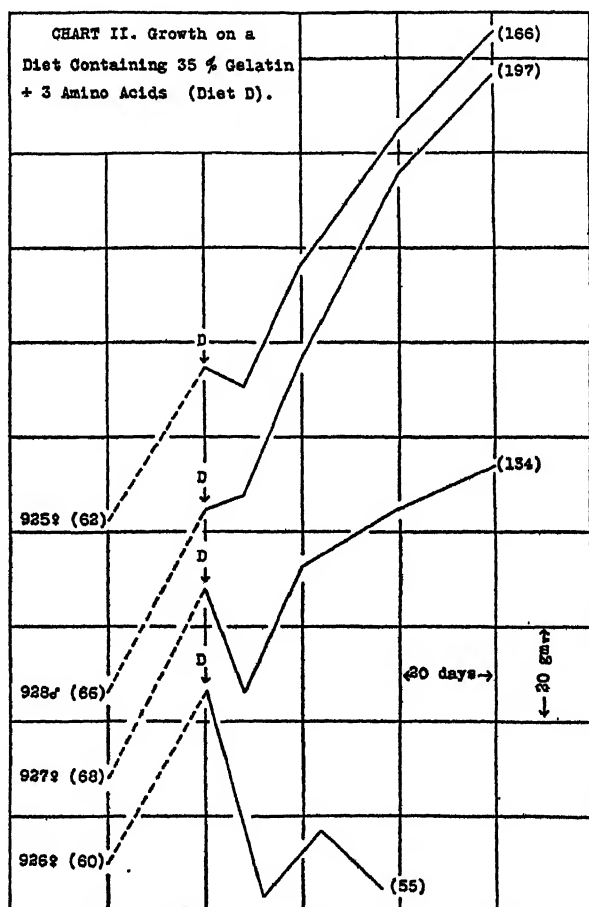


CHART II.

926 and 927, Chart II) underwent profound losses, but Rat 927 partially recovered later. Rats 925 and 928 experienced only slight decreases in body weight followed by almost normal growth. The latter animals, though constituting a small percentage of the

total number employed, are of particular significance in that they prove that very good growth *is possible* when gelatin supplemented with tyrosine, cystine, and tryptophane serves as the sole source of nitrogen except for the small quantity present in 200 mg. of yeast. On the other hand, it is quite evident that Diet D permitted rapid increases in weight in *only two out of a total of twenty-six animals*. This fact led us to try the influence of other proportions of gelatin, and of other supplementing amino acids.

*Growth on Gelatin Diets Supplemented with Seven and Eleven Amino Acids.*—Inasmuch as tyrosine, cystine, and tryptophane, as stated above, are not the only factors which are low in gelatin diets, attempts were made to improve the quality of the protein by the further addition of histidine, valine, phenylalanine, and glutamic acid. For this purpose, two diets were formulated (Diets F and G, Table I) containing 30 and 25 per cent of gelatin respectively. The data in Table II show that presumably each contained adequate amounts of all of the amino acids definitely known to be present in casein with the exception of isoleucine and hydroxyglutamic acid. Since the latter may be disregarded for reasons already stated, the absence of isoleucine is the only possible deficiency in the rations in so far as our knowledge of the composition of gelatin and casein permits one to judge.

Four rats were placed upon each of these diets after preliminary periods upon Diet A. To our surprise the growth curves were not better than those obtained with Diet D. Of the eight animals two died within 16 days. The experiments upon three others were discontinued after the animals had lost weight very rapidly, and were in a greatly weakened condition. Three recovered from the injurious effects of the gelatin and made slow gains. One of these (Rat 140) was subsequently changed to Diet D and has already been referred to in connection with Chart I. The graph makes evident the fact that the growth of this animal was not altered significantly by the transfer from Diet F containing seven supplementing amino acids to Diet D containing only three. The growth curves of the other animals upon Diets F and G (with the exception of Rat 141) are omitted from the charts as they add nothing to the solution of the problem in question.

Rat 141 was changed from Diet F to Diet H containing 15 per cent of gelatin supplemented with eleven amino acids. An

inspection of Table I reveals the fact that in addition to the seven amino acids included in Diet F, Diet H contained the three leucines and aspartic acid. The growth curve is shown in Chart III. The animal made a slow but fairly consistent gain upon this ration, and when the experiment was discontinued had increased in body weight to the extent of 24 gm. in 72 days. At a later

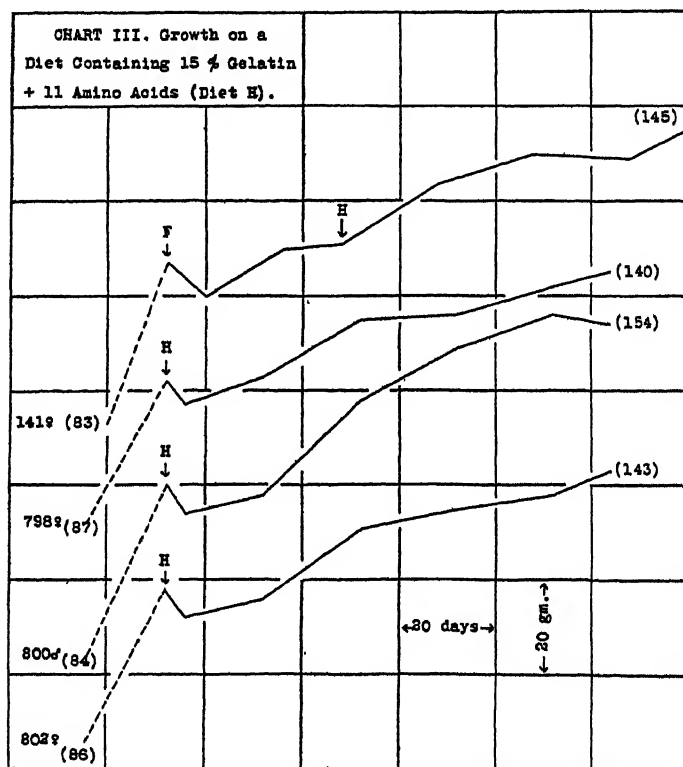


CHART III.

date, three other rats (Rats 798, 800, and 802) of a single litter were transferred directly from the casein ration to Diet H. The growth curves of these animals also are shown in Chart III, and manifest increases in body weight comparable to that attained by Rat 141. The only striking difference between these experiments and those involving the use of the 35 per cent gelatin diet is the fact

that upon the lower level of gelatin none of the animals succumbed. After becoming "adjusted" to Diet H, all of the rats made moderate gains. It should be emphasized that Diet H contained all known amino acids with the exception of hydroxyglutamic acid. Aside from the latter, the only component present in distinctly lower quantity than in an 18 per cent casein diet is lysine. That this was not a limiting factor is made probable by the observation of Osborne and Mendel (1915) that edestin, which contains 1.65 per cent of lysine, supports normal growth when included in the ration at a 15 per cent level. Under such circumstances, 100 gm. of the edestin diet carry only 0.25 gm. of lysine, or less than one-third the amount present in our Diet H.

Attempts to improve the quality of gelatin rations by increasing the proportion of the protein gave even less satisfactory results than were secured with 15 to 35 per cent levels. Of three rats placed upon a diet containing 55 per cent of gelatin supplemented with tyrosine, cystine, and tryptophane, two died promptly after very rapid losses in weight, and the third was moribund when the experiment was discontinued. Further studies of this sort seemed very unpromising and were abandoned.

*Does Gelatin Exert a Deleterious Action?*—In order to determine whether gelatin, or the unusually large quantities of glycine and alanine which it contains, is detrimental to the organism two modifications of Diet A were formulated, one containing 9 per cent of glycine and 6 per cent of racemic alanine (Diet B), and the other containing 35 per cent of gelatin (Diet C). The added components replaced equivalent amounts of carbohydrate without altering the ratio of casein. Thus each ration carried an adequate quantity of protein of suitable quality (casein) in addition to the superfluous nitrogenous materials. After preliminary periods upon Diet A, two rats were placed upon Diet B, and three upon Diet C. The results are shown in Chart IV. All five animals, but especially those upon Diet C, exhibit decreases in growth rates after the transfer from the normal ration. Rat 188, one of those which received the diet containing 35 per cent of gelatin, experienced a loss in weight of 17 gm. in 8 days before becoming adjusted to the new dietary régime. Following recovery, rather rapid growth ensued in all cases. The experiments indicate that neither gelatin nor a mixture of two of its component

amino acids permanently prevents growth when incorporated in an otherwise normal diet. On the other hand, the large proportion of gelatin (35 per cent) may produce a *temporary inhibition*

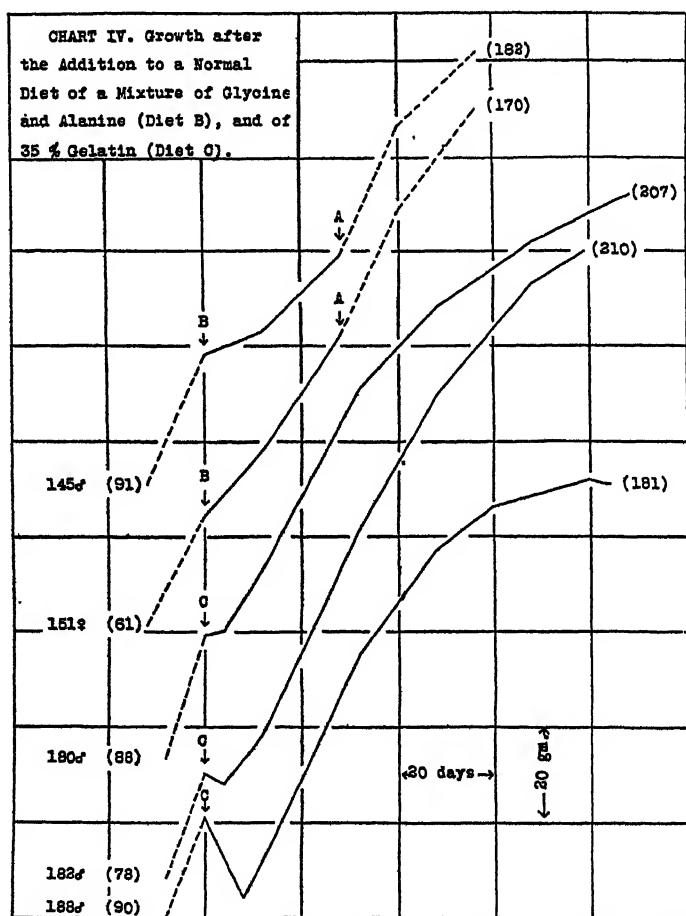


CHART IV.

in growth despite the presence of a suitable amount of adequate protein. This fact, when considered in conjunction with the observation that renal injury frequently followed the administration of the high gelatin diet (Diet D), renders it difficult to

escape the impression that this protein, at the level indicated, may be positively detrimental to as well as negatively inadequate for the organism. Whether this suggested deleterious action is a characteristic of gelatin alone or of other proteins also when taken in excessive amounts (*cf.* Newburgh and associates, 1925) remains to be proved. It should be recalled, however, that Osborne and Mendel (1924) have secured excellent growth, without renal changes other than hypertrophy, with diets containing 95 per cent of protein.

*Growth upon Diets of Hydrolyzed Gelatin Supplemented with Three Amino Acids.*—In a further attempt to arrive at an explanation for nutritive failure of animals upon gelatin diets, we have reinvestigated the influence of complete hydrolysis of the protein preliminary to its incorporation in the ration. The method of hydrolysis has already been outlined. Two diets were employed. Diet E contained 35 per cent of hydrolyzed gelatin and was exactly analogous in composition to Diet D. Diet I contained 15 per cent of the hydrolyzed protein and is comparable to Diet H. After the usual preliminary periods upon Diet A, three rats were transferred to Diet E and four to Diet I. The results are shown in Chart V. As will be observed, the change from the control to the hydrolyzed gelatin rations was followed by the usual losses in weight. After 12 to 16 days upon Diet E, and 4 to 12 days upon Diet I, recovery appeared to occur, and was succeeded by slow growth until the experiments were discontinued. At the expiration of 80 days upon Diet E, Rat 195 weighed 1 gm. less than when transferred to this ration. The net gains of Rats 192 and 194, upon the same diet, were 10 and 12 gm. respectively.

The four rats upon Diet I made somewhat better net gains. However, this difference is due primarily to the fact that, like the animals upon the unhydrolyzed protein, those which received the higher level of hydrolyzed gelatin usually experienced the greater initial losses in weight.

Although no mortality occurred among the seven animals upon these diets (a fact which may or may not be of significance in connection with the suggested toxicity of unhydrolyzed gelatin), nevertheless the growth curves indicate that hydrolysis occasions very little if any improvement in the growth-promoting properties of this protein (*cf.* Charts I and II with Chart V). It appears

that the inadequacy of gelatin as a source of nitrogen is not to be ascribed to the presence of amino acid linkages which cannot be severed by the digestive fluids of the gastrointestinal tract. In

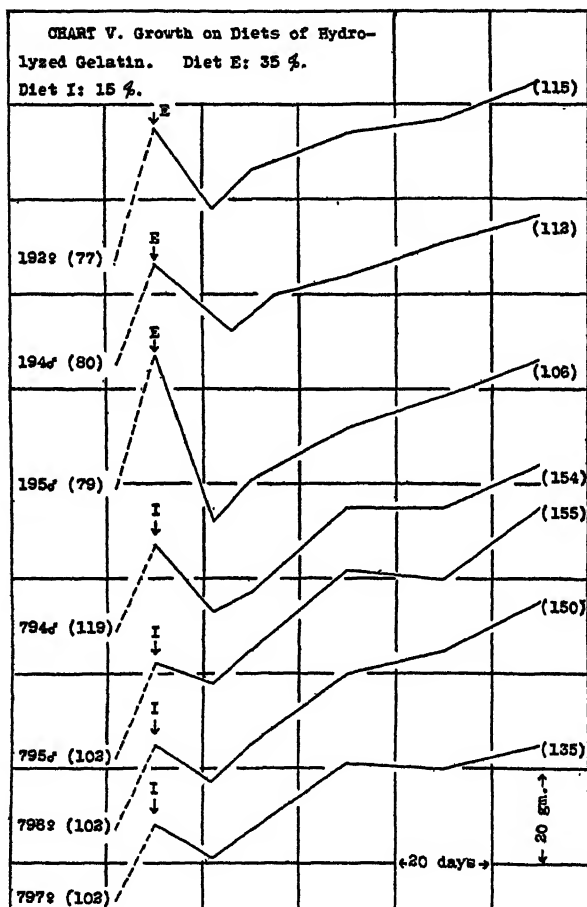


CHART V.

this respect our results are not in accord with those of Totani (1916).

*Food Consumption of the Experimental Animals.*—In Table III are presented the values representing the average daily food con-

sumption and average daily change in body weight of each rat. For convenience these data are arranged to correspond to the charts.

TABLE III.

*Food Consumption and Body Weight Changes of the Experimental Animals.*

Chart No.	Rat No.	Diet.	Average daily change in body weight.	Average daily food consumption.	Chart No.	Rat No.	Diet.	Average daily change in body weight.	Average daily food consumption.
			gm.	gm.				gm.	gm.
I	138	A	+2.8	7.8	IV	145	A	+2.3	8.7
		D	-1.9	3.5			B	+0.8	7.0
	139	A	+2.2	7.8		151	A	+1.5	9.0
		D	0	5.3			"	+1.9	5.7
	140	A	+2.5	7.0		180	B	+1.4	5.4
		F	+0.4	6.7			A	+1.7	8.0
	150	D	+0.2	6.3		182	"	+3.3	7.6
		A	+2.7	5.9			C	+1.1	6.6
	169	D	-2.3	3.5		188	A	+2.8	6.4
		A	+4.9	10.0			C	+1.3	6.0
II	925	D	0	5.1	V	192	A	+2.8	7.5
		D	0	5.1			C	+0.8	5.6
	926	A	+1.7	5.9		194	A	+3.5	7.3
		D	+1.2	6.0			E	+0.1	4.2
	927	A	+1.8	6.9		195	A	+2.6	6.0
		D	-1.1	3.1			E	+0.1	3.7
	928	A	+2.0	6.7		794	A	+3.5	7.9
		D	+0.4	6.2			E	0	3.8
	928	A	+2.0	6.0		795	A	+2.3	7.1
		D	+1.5	6.5			I	+0.2	8.1
III	141	A	+2.8	8.5	796	795	A	+0.2	8.1
		F	+0.1	6.0			A	+2.5	8.3
	798	H	+0.3	5.6		796	I	+0.4	7.6
		A	+1.9	6.4			A	+2.3	8.3
	800	H	+0.3	7.4		797	I	+0.4	7.9
		A	+2.3	6.5			A	+2.0	8.3
	802	H	+0.4	7.2			I	+0.2	7.5
		A	+2.0	6.8					
	802	H	+0.3	7.3					
		H	+0.3	7.3					

While admittedly the interpretation of food intakes is sometimes a difficult question, we believe the figures recorded in Table III indicate that the *amount* of food consumed by the individual



animals was not the factor which *determined* whether an increase in weight did or did not occur. It cannot be denied that in some of the experiments a rough correlation between food ingestion and rate of growth appears to exist (*cf.* Rats 138, 150, and 926). On the other hand, with many of the animals the intake upon the gelatin diets was almost as large as upon the casein ration, and in some cases even larger. Rat 140 consumed an average of 7.0 gm. of Diet A daily, and 6.7 and 6.3 gm. respectively of Diets F and D. Thus decreases of approximately 4 and 10 per cent in food ingestion were accompanied by decreases of 84 and 92 per cent in growth rates. Rat 927, with a decrease of a little more than 7 per cent in food intake, showed an 80 per cent diminution in growth rate. Other animals, particularly those upon the lower levels of gelatin (Rats 794, 798, 800, 802), ingested just as much of the experimental rations as of the control diet. Both our experience and the observations of others with food mixtures highly deficient in one or more amino acids have taught us to expect very pronounced failure in appetite when the diets are wanting in some factor or factors essential for the growth process. The high level at which many of our animals consumed the gelatin rations leads us to believe that the supplemented mixtures employed in this investigation were not inadequate *to a pronounced degree* in any necessary amino acid. Probably the diminished food intakes of some of the rats upon the diets containing 35 per cent of gelatin may be attributed to the renal involvement.

It should be emphasized also that average figures, such as are recorded in Table III, may lead to erroneous conclusions in that they do not indicate the *progress* of the decreases in food consumption. This point is of considerable importance in arriving at a correct interpretation. An illustration will serve to make this fact clear. Rat 150, one of those in which the failure in growth might be attributed to the diminished food intake, showed an average daily food consumption of 5.9 gm. on Diet A, and 3.5 gm. upon Diet D (*cf.* Table III). Upon the latter ration the average daily loss in weight (2.3 gm.) was greater than was experienced by any of the other animals. Our records indicate, however, that *during the first 4 days upon Diet D, the daily food ingestion averaged 7.2 gm., or was considerably larger than upon the normal casein diet. During the same 4 day period the animal lost an average*

of 1.5 gm. daily. In the three subsequent 4 day periods preceding death, the average daily food consumption amounted to 4.2, 1.8, and 0.8 gm. respectively, accompanied by an increasingly rapid decrease in body weight. In other words, as was observed several years ago by Hopkins (1912) in deficiency studies, the failure in appetite *followed* the failure in growth. For reasons already stated, we are inclined to the belief that renal involvement rather than an amino acid deficiency was the immediate cause of death in many of our experiments, including that upon Rat 150. But whatever may have occasioned the fatal outcome, certainly the initial loss in weight of Rat 150 upon Diet D was not due to insufficient ingestion of food. In a later communication we shall present additional information concerning the relation of food composition to appetite.

#### SUMMARY.

The above results indicate that diets containing gelatin as the sole protein, at levels of 15 to 55 per cent, are not suitable sources of nitrogen even when supplemented with the amino acids known to be missing, or present in relatively small amounts. About half of the total number of rats placed upon a 35 per cent gelatin diet to which had been added appropriate quantities of cystine, tyrosine, and tryptophane, successfully weathered the change; and after periods of more or less profound "adjustment," maintained body weight or even grew at varying rates.

Reducing the level of gelatin from 35 per cent to 15 per cent of the diet, and incorporating the eleven amino acids which, on the basis of the best available analyses, seem to be the most likely possible sources of deficiency, failed to render the food more satisfactory for growth. It appears that valine and isoleucine, which presumably are absent from gelatin, are not limiting factors in the nutritive properties of this protein. Nor did the increased proportions of phenylalanine, aspartic acid, glutamic acid, and histidine in the supplemented 15 per cent gelatin ration contribute beneficially to the nutritive condition of the animals.

Attempts to improve the quality of gelatin are complicated by what appears to be a deleterious action exerted by the protein when supplied at a 35 per cent level, as manifested by the early fatal outcome of many of the experiments, and the frequent

incidence of severe renal injury. A temporary inhibition in the growth rate of rats upon casein diets may be induced by substituting gelatin for 35 per cent of the carbohydrate.

Diets containing hydrolyzed gelatin supplemented with amino acids do not support growth more successfully than do similar rations containing unhydrolyzed gelatin. Evidently, the peculiar inadequacies of this material for growth purposes are not to be ascribed to the presence in the molecule of linkages which cannot be severed by the digestive mechanism.

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## ON THE ISOELECTRIC PRECIPITATION OF PEPSIN. II.

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Company, Chicago.)*

(Received for publication, September 1, 1928.)

In a previous paper (1), it was shown that pepsin of high proteolytic power can be obtained by isoelectric precipitation provided the materials are handled rapidly and at low temperatures. Fractions were obtained at pH values ranging from 2.4 to 3.85. The fraction precipitating out at pH 2.4 to 2.5 was shown to have the highest proteolytic activity. This work is an attempt further to purify pepsin by introducing several refinements into the method and to establish the chemical relationship between the various fractions which are obtained. A long series of experiments was carried out during the past year with different solvents, *etc.*, in an attempt to increase the proteolytic properties of the enzyme fractions. We were not able, however, to separate a single sample of pepsin which was superior in digestive power to those already reported. To our knowledge the proteolytic activity of the pH 2.4 to 2.5 fraction is the highest reported by any investigator when assayed by an official and recognized method. We feel that we are dealing with the enzyme in the form in which it naturally occurs in the glandular mucosa of the stomach.

### *Method of Preparation and Purification.*

The method of Fenger and Andrew (1) was employed for the separation and purification of the pepsin. The collection of the stomach linings was started in October and continued through December. These were handled as previously described, and precipitates were collected at pH values of 2.5, 3.0, and 3.8. In order further to purify the fractions and to remove all traces of

ash, they were repeatedly washed in dilute solutions of hydrochloric acid at a hydrogen ion concentration corresponding to the pH value at which the fraction was collected. Practically all impurities should be removed by this process and a purer product obtained than when this procedure is omitted. This step is similar to that used by Field (2) in the preparation of ash-free gelatin. An ample supply of each fraction was prepared.

*Properties of the Various Fractions.*

All three fractions consisted of white, granular powder, nearly insoluble in distilled water but slowly and completely soluble in acidified water. Determinations of the ash content showed the fractions precipitated at pH 2.5 to be ash-free, while the other two fractions separated at pH 3.0 and pH 3.8 contained 0.025 and 0.010 per cent of ash respectively. The ash determinations were made on 1 gm. samples. The powdered pepsin was placed in platinum crucibles and heated in an electric muffle at temperatures just below dull red heat (500°) in order to prevent volatilization of inorganic chlorides.

The proteolytic activity as determined by the United States Pharmacopoeia assay (3) was as follows:

pH	Maximum.	Minimum.
2.5	1:65,000	1:50,000
3.0	1:50,000	1:42,500
3.8	1:45,000	1:40,000

The highest testing fraction is consistently obtained at pH 2.5, confirming our previous results. Forbes (4) likewise found the isoelectric point of two samples of purified pepsin to lie at 2.5 on the pH scale. The existence of pepsin fractions of high proteolytic activity and precipitating at higher pH values is, however, evidenced by the above.

With the view of determining the chemical difference existing between these three fractions, complete analyses were made of composite samples of the three precipitates. The results given in Table I were obtained.

The carbon and hydrogen were determined by combustion, the nitrogen by the Gunning-Kjeldahl method, and the phosphorus weighed as magnesium pyrophosphate. Sulfur and chlorine were determined by the method of Carius.

From the tabulated data it will be seen that some striking differences exist in the composition of the three fractions. The absence of chlorine in the pH 2.5 fraction is significant. The chlorine in the pH 3.0 and 3.8 fractions is, of course, organically bound. The percentage of phosphorus increases with the proteolytic activity of the various fractions, while the percentage of

TABLE I.

	Enzyme fraction precipitated at:		
	pH 2.5.	pH 3.0.	pH 3.8.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Carbon.....	53.15	52.27	51.49
Hydrogen.....	7.20	6.93	7.03
Nitrogen.....	14.43	14.62	14.43
Sulfur.....	0.88	0.96	1.08
Phosphorus.....	0.13	0.11	0.08
Chlorine.....	0	0.96	1.02

TABLE II.

	Enzyme fraction precipitated at:					
	pH 2.5.		pH 3.0.		pH 3.8.	
	Nitrogen.		Nitrogen.		Nitrogen.	
	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
Total N.....	430.25	100.00	438.62	100.00	230.68	100.00
Amide ".....	42.95	9.98	38.46	8.77	26.10	9.29
Humin ".....	26.10	6.06	23.83	5.44	17.16	6.12
Arginine N.....	8.97	2.05	8.12	1.86	7.85	2.79
Histidine ".....	11.13	2.59	12.61	2.89	12.12	4.32
Lysine N.....	10.30	2.38	6.93	1.35	6.07	2.22
Non-amino N.....	15.31	3.56	22.81	5.20	15.38	5.47
Monoamino ".....	304.85	70.85	308.46	70.08	189.66	67.53

sulfur decreases. Both these elements are part of the enzyme molecule. This is contrary to Pekarharing and Ringer's (5) contention that the phosphorus is an impurity.

In order to establish further relationship between the fractions the nitrogen distribution was determined by the method of Van Slyke (6).

The results are shown in Table II.

All three samples are high in monoamino nitrogen, and low in

histidine, arginine, and lysine. The sample precipitating at pH 3.8 is higher in arginine and histidine than the other two samples. The non-amino nitrogen is lowest in the pH 2.5 and highest in the pH 3.8 sample, while in the case of the monoamino nitrogen the reverse holds true. In general it may be said, however, that no striking differences occur in the nitrogen distribution of the three samples save for the above exceptions. All three samples reacted positively to Millon's reagent, the xanthoproteic test, and gave strong biuret reactions.

All these analyses are typical of a protein. The first fraction precipitated at pH 2.5 evidently is the enzyme protein, while the pH 3.0 and 3.8 fractions represent early stages of peptic decomposition with progressively decreasing proteolytic activities. Subsequent enzyme fractions remaining in solution at pH values above 3.8 have lost their identity to such an extent that they no longer are able to become isoelectric. These fractions still possess proteolytic properties until they reach the stage when they are sufficiently small to diffuse through parchment or animal membranes. The gradual decrease of proteolytic activity of the enzyme itself is paralleled by loss of its complex protein characteristics.

#### CONCLUSIONS.

An ash-free pepsin has been obtained with an isoelectric point of 2.5 on the pH scale, and pepsin containing a slight amount of ash has been precipitated at pH 3.0 and 3.8.

Complete analyses have been made of these pepsins and their digestive power determined. The proteolytic activity decreases with increase in pH at the isoelectric point. The analyses in all three cases are typical of a protein.

The authors desire to express their gratitude to Dr. J. J. Vollertsen for valuable assistance and helpful cooperation in carrying out this investigation.

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## THE ALKALINE DECOMPOSITION OF CYSTINE.

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(Received for publication, August 20, 1928.)

For many years it has been customary to classify biological sulfur into "easily split sulfur" and a form which was less easily split. The basis for this distinction has been the test in which the sulfur-containing compound is boiled with lead acetate in an excess of alkali. Under these conditions a part of the sulfur quickly splits off as  $S^{2-}$  and, reacting with the  $PbO_2^{2-}$ , precipitates lead sulfide. Thus the amount of lead sulfide produced was regarded as a measure of the proportion of easily split sulfur present. This reaction has been of special interest because of the conclusions to which it has led concerning the state of sulfur in the protein molecule. Various investigators have obtained from 52 to 83 per cent of the sulfur of cystine as lead sulfide while the proportion obtained from different proteins has varied from about 10 to nearly 100 per cent. A good bibliography of the earlier work in this field has already been published by Hoffman and Gortner (1).

A perusal of this literature shows that slight variations in the conditions of the test have resulted in wide differences in the yield of lead sulfide obtained from even cystine itself, while the more recent studies of Bergmann and Stather (2), Brand and Sandberg (3), and others have demonstrated the great differences in stability of various biological sulfur compounds as regards this reaction.

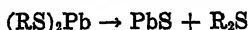
Inspection of the data obtained by different investigators on the yield of lead sulfide from cystine reveals one very suggestive point: The different percentages reported are nearly all very close to small whole number ratios which suggest their origin from some definite reaction. For example, we have 83 per cent (5:1), 68 per cent (2:1), and 53 per cent (1:1). This last figure, by Schulz (4), resulted from an attempt to raise and reconcile the figures of other workers. He argued that the long heating with alkali afforded too much opportunity for oxidation of the sulfur and to avoid this,



he added metallic zinc to the alkaline solution. The result was a *decrease* in the yield to a little over 50 per cent. A plausible explanation of this ratio would seem to be that the addition of zinc reduced the cystine, in great part, to cysteine and that the latter reacted in the alkaline lead solution to form the lead salt as follows:



which on being heated decomposes as follows:



This reaction, well known for other mercaptans (Klason (5)) explains the 1:1 partition of the sulfur, while the figure of 53 per cent, as opposed to 50 per cent, is no doubt the result of incomplete reduction by the zinc.

The complicated nature of the alkaline decomposition of cystine is evidenced by the variety of changes involved: The optical activity of the cystine decreases rapidly, sulfide ion is formed, and, in presence of plumbite precipitates as lead sulfide, cysteine is formed, and ammonia is evolved. It is the purpose of this paper to record the results of investigations of some of the various reactions involved.

Qualitatively speaking, the effect of the alkali is to cause gradual decomposition of the cystine with formation of sulfide ions from a part of the sulfur of the decomposed portion. The sulfide is of course, in the presence of the plumbite, precipitated as lead sulfide, but in the absence of plumbite it accumulates and causes reduction of some of the undecomposed cystine to cysteine. The free sulfur resulting reacts with some of the unoxidized sulfide to form polysulfide, and as such, gradually reacts with the alkali in the usual way.

The general practice of heating the cystine for some hours in the solution of alkaline plumbite accelerates these reactions to the extent of making an individual scrutiny of them impossible. Most of the present studies were carried out at about 25°. The lower temperature makes it possible to follow more completely the course of the individual reactions.

#### EXPERIMENTAL.

##### *Yield of Lead Sulfide.*

Some preliminary experiments were carried out to determine such points as the effect of air exposure and time of contact of plumbite on the yield of lead sulfide. The following is typical of

the results obtained. Into each of four Erlenmeyer flasks (Flasks A, B, C, and D) were inserted 100 cc. of 4.00 M NaOH and 1.000 gm. of pure cystine. To Flasks A and C were added at once 10 gm. of  $(\text{CH}_3\text{COO})_2\text{Pb} \cdot 3\text{H}_2\text{O}$  and all four flasks were allowed to stand at room temperature for 24 hours with Flasks A and B slowly swept with pure nitrogen and Flasks C and D exposed to the air. At the end of the 24 hours, the same amount of lead acetate was added to Flasks B and D, all were allowed to stand 1 hour longer, after which the lead sulfide from each was filtered on a weighed Gooch crucible, washed with water and alcohol, and dried in a desiccator to constant weight. Table I shows the results.

While the filtrates from all four flasks after the first filtration were perfectly clear, those from Flasks A and C (lead added at

TABLE I.

*Yield of PbS Obtained from Alkaline Cystine Solutions Containing Lead.*

After standing:	1 day.		5 days.		11 days.	
Flasks.	Weight of PbS.	Per cent of S as PbS.	Total weight of PbS.	Total per cent S as PbS.	Total weight of PbS.	Total per cent S as PbS.
	<i>gm.</i>		<i>gm.</i>		<i>gm.</i>	
A	0.3738	18.8	1.3953	70.1	1.5231	76.5
B	0.1019	5.1	1.4098	70.8	1.5004	75.3
C	0.3215	16.1	1.0814	54.3	No further precipitate.	
D	0.0804	4.0	1.3826	69.4	1.4101	70.8

once) were practically colorless while those from Flasks B and D were quite yellow. Evidently the loss of lead sulfide in these cases is to be ascribed to oxidation of the sulfide to polysulfide. Such solutions when acidified always gave a heavy precipitate of free sulfur. On standing these filtrates darkened within 15 minutes at room temperature. They were then allowed to stand 4 days longer under the same conditions of exposure to air as before and the additional lead sulfide was again determined (see Table I, 5 day series). The same process was repeated after 6 days more (a total of 11 days). After this, only negligible amounts of lead sulfide were formed. The failure of Flask C to continue to produce lead sulfide in this series is yet to be explained. In other similar experiments the lead sulfide produced under such condi-

tions attained a total figure between that of Flasks B and D. Otherwise the experiment shows the effect of atmospheric oxidation in reducing the yield as well as the necessity of keeping the plumbite in constant contact with the solution in order to remove the sulfide ion as fast as it is formed and before it can be reoxidized.

A similar series which partly duplicates some of the conditions in the above and also furnishes some information as to the effect of reacidifying before removing lead sulfide is shown in Table II. Four flasks were employed. Flasks, A, B, and C were continually swept with nitrogen while Flask D was exposed to air. The proportions of cystine, alkali, and lead acetate duplicated those in Table I. Flask A was treated exactly as was Flask A in Table I.

TABLE II.

*Yield of PbS Obtained from Alkaline Cystine Solutions Containing Lead.  
14 Days Standing.*

Flask.	Weight of PbS.	Per cent S as PbS.
	<i>gm.</i>	
A	1.5716	78.9
B	1.5420	77.4
C	0.1660	8.3
D	1.5320	76.9

Flask B was treated the same except that the solution was acidified with 100 cc. of 8 M acetic acid just before filtering. Flask C was treated with the lead acetate only 1 hour before filtering the lead sulfide (similar to Flask B in Table I). Flask D was treated as was Flask C in Table I; the lead acetate was added at once and full exposure to air was allowed. All four flasks stood at room temperature for 14 days.

The results, in general, confirm those of Table I. It is evident that acidifying the solution, even though it increases the concentration of  $Pb^{++}$ , does not result in any increase in the yield of lead sulfide. As in Table I, the necessity of having the plumbite constantly in the solution is evidenced while with Flask D a figure was obtained of an order which should have resulted in Flask C, Table I.

*Potentials of Plumbite Solutions.*

As shown above, a high yield of lead sulfide requires the presence of plumbite during the whole time of decomposition of the cystine. Since this supports the transitory character of the sulfide ion it becomes of importance to know something of the maximum level of concentration which sulfide ion can attain in the presence of various concentrations of alkali and plumbite. The usual value for the solubility product of lead sulfide being assumed to hold here ( $K = 4 \times 10^{-28}$ ) the sulfide ion concentration depends on the concentration of  $\text{Pb}^{++}$  furnished by the equilibrium:

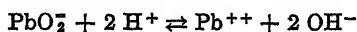


TABLE III.

*Potentials Obtained from Half-Cells of Lead in Sodium Hydroxide-Sodium Plumbite Solutions with Corresponding Concentrations of  $\text{Pb}^{++}$  and  $\text{S}^{--}$ .*

Normality of NaOH.	Potential of Pb cell volts.	Concentration of $\text{Pb}^{++}$ .	Corresponding concentration of $\text{S}^{--}$ .
<i>N</i>			
4.00	0.3129	$8.1 \times 10^{-16}$	$5.0 \times 10^{-13}$
2.00	0.2911	$4.4 \times 10^{-15}$	$9.1 \times 10^{-14}$
1.00	0.2690	$2.5 \times 10^{-14}$	$1.6 \times 10^{-14}$

To determine this, potentials were measured between metallic lead and alkali of various concentrations, saturated with lead hydroxide. Saturated calomel half-cells were used with an absolute potential (at 25°) of +0.5234 volts. The formula

$$E = 0.0295 \log \frac{C}{K}$$

was employed.  $E$  = the potential of the lead half-cell,  $K$  the solution tension of metallic lead ( $3.3 \times 10^{-5}$ ) and  $C$  the concentration of  $\text{Pb}^{++}$ . Table III shows the average results obtained.

The figures in Table III show the order of completeness of sulfide ion removal from such solutions. Even when the lack of correction for boundary potentials in the cell and the uncertainty of the solubility product of lead sulfide in such concentrated solutions are admitted, there seems to be no doubt as to the quantitative removal of sulfide when excess plumbite is present.

*Optical Activity.*

The optical activity of alkaline cystine solutions may be used both as a criterion of the amount of racemization caused by the alkali and also of the amount of reduction of cystine. While strong alkali has its effect in racemizing cystine, a fair proportion can be recovered with its optical activity practically unchanged, particularly when the solution has not been heated. For example, a solution of 1.5 gm. of cystine ( $[\alpha]_D = -207.0^\circ$ ) per 100 cc. of 4.0 M NaOH stood for 20 days under nitrogen at room temperature. At the end of that time about 0.2 gm. of cystine was recovered, which, under the microscope, was seen to contain some crystals of racemic cystine but which had a specific rotation of  $-200^\circ$ . However, this figure does not represent the true proportion of *l*- to racemic cystine since the purification process removes a larger proportion of the racemic than of the *l* form (6).

Table IV shows the effect of strong alkali on the rotation of a sample of cystine  $[\alpha]_D = -207.0^\circ$ . The solution used contained 10.00 gm. of cystine in 500 cc. of 4.0 M NaOH. This solution was allowed to stand at room temperature under pure nitrogen and at intervals 25 cc. aliquots were measured into 50 cc. volumetric flasks and made up to volume with 6.0 M HCl. The result was a solution corresponding to 1.00 gm. of cystine per 100 cc. in 1.0 M HCl and containing also 2.0 M NaCl. The change in conditions, had there been no racemization or reduction, would, because of the sodium chloride, have lowered the  $[\alpha]_D$  value to about  $-191^\circ$  (7). After cooling, this solution was adjusted to the mark and filtered into a 4 dm. polariscope tube. Rotations were taken at  $29^\circ$ .

After the last aliquots described in Table IV were removed, further aliquots were aerated before rotations were taken. The result was a rise in  $[\alpha]_D$  to values as high as  $-90^\circ$ . It is evident that the change of cystine to cysteine is responsible for a part of the decrease in activity.

This conclusion was confirmed by a number of other experiments. For example, the decrease in activity is markedly accelerated when the above described experiment was duplicated with the addition of sodium sulfide to the cystine solution. The specific rotation, taken at once, showed a drop to about  $-50^\circ$  while the sample on being brought to about pH 8 and treated for some hours with a stream of oxygen rose to over  $-100^\circ$ . Subse-

quent samples gave similar results. In such an experiment the greater proportion of cysteine formed made the rotation much more sensitive to contact with slight amounts of oxygen. Acidification of the samples also gave very heavy sulfur precipitates from the polysulfide formed and complete removal of the colloidal sulfur was necessary before rotations could be read.

On the other hand, if the concentration of sulfide be kept low by the presence of excess plumbite the loss in optical activity is again accelerated but here the loss is due to practically complete decomposition of the cystine. Owing to the presence of less cysteine when plumbite is present (see below), reoxidation produces little or no activity.

TABLE IV.

*Specific Rotation of an Alkaline Solution of Cystine. Room Temperature.*

Time.	Specific rotation.	Time.	Specific rotation.
hrs.	degrees	hrs.	degrees
0	-191	6	-131.8
$\frac{1}{2}$	-181.2	7	-128.0
1	-161.5	23	-88.0
2	-151.0	27	-81.0
3	-142.5	31	-76.5
4	-138.0	47	-51.5

For example, in Table II, the filtrates from all four flasks were examined polariscopically. Those from Flasks A, B, and D were completely inactive. Flask C showed an observed rotation in a 4 dm. tube of  $-3.04^\circ$ . For this, calculated to the original 1.00 gm. of cystine,  $[\alpha]_D = -76.0^\circ$ .

In several experiments (see below) on the rate of loss of cystine and formation of sulfide, polariscopic examinations were made of the unoxidized filtrate from the hydrogen sulfide determinations. For example, a solution of 1.5 gm. of cystine ( $[\alpha]_D = -207^\circ$ ) per 100 cc. of 4.0 M NaOH, after standing in the presence of excess plumbite at room temperature for 3 weeks gave an optically inactive filtrate. Duplication of these conditions but without the plumbite gave a solution with an observed rotation of  $-0.91^\circ$  which, on aeration of the solution was raised to  $-1.22^\circ$  (both figures calculated to the original concentration of the solution).

Calculated to an original concentration of 1.5 gm. of cystine per 100 cc. (regardless of the increased concentration indicated by Folin-Looney figures) this indicates that about 90 per cent of the cystine had been racemized. Several other similar experiments gave figures of the same order for the degree of racemization. In the absence of any other information it is to be assumed that racemization is proportionally distributed between the oxidized and the reduced forms.

#### *Concentration of Cystine and Cysteine.*

In order to follow the changes in cystine and cysteine concentration several series of experiments were made in which samples of pure cystine dissolved in sodium hydroxide were preserved for some months at room temperature and aliquots were withdrawn at intervals for analysis. A number of preliminary trials had demonstrated the important influence of atmospheric oxygen on the course of the reaction. The sulfide formed from the cystine is susceptible to oxidation, if allowed to accumulate in the absence of lead, while the cysteine formed is, in alkaline solution, very much more unstable and easily oxidized. The solutions of cystine in sodium hydroxide were therefore made up under nitrogen. They were preserved in Erlenmeyer flasks equipped with inlet and outlet tubes and were overlaid with a thick layer of paraffin oil. The space above the oil was swept with pure nitrogen and the flasks sealed. The withdrawal of samples was always carried out under nitrogen and after each withdrawal the flask was thoroughly swept again.

The stream of nitrogen was purified, first by passage through alkaline pyrogallol and then through a long silica tube filled with copper shavings and heated. A wash bottle of reduced indigo carmine solution, kept constantly in the train, showed no evidence that any oxygen escaped removal.

The determination of cystine and cysteine was made by the Folin-Looney procedure. Since complete removal of sulfide was necessary before the Folin-Looney reagent could be applied the following procedure was used.

A 250 cc. volumetric flask with short neck, containing the necessary excess of hydrochloric acid, was placed in a vacuum desiccator. From the lid of the desiccator there dipped into the

flask a capillary glass tube connected to the supply of pure nitrogen. The desiccator was then evacuated while a very small stream of nitrogen was carried through the flask. The low pressure inside the desiccator made very efficient sweeping possible. When the flask had been thoroughly swept, a 5 cc. aliquot of the alkaline cystine solution was quickly introduced under the acid and the sweeping continued until all sulfide was removed. Tests on both alkaline cystine and sodium sulfide solutions showed that 2 hours sweeping was ample to reduce the  $\text{H}_2\text{S}$  below any amount which could reduce the Folin-Looney reagent. When sweeping was complete the solution was made up to volume and aliquots for cystine and cysteine were removed and run by the usual Folin-Looney procedure (8).

As indicated by the data in Table V, the concentration of alkali used was varied from 0.1 M to 4.0 M and for each concentration, series were run both with and without lead. In absence of lead, the determination of cystine and cysteine was accompanied by the determination of hydrogen sulfide. The results of the  $\text{H}_2\text{S}$  determinations are also included in Table V and are discussed below under "Production of Sulfide." Cystine of  $[\alpha]_D = -209.0^\circ$  was used.

It is evident that in the removal of cystine by the alkali the concentration of the latter plays a very minor part in comparison to the effect of the presence or absence of lead. It is also to be noted that in the 4.0 M NaOH series without lead the apparently very rapid rise in cysteine concentration, as indicated by the Folin-Looney method, brings the sum of the cystine + cysteine to considerably above the amount of cystine originally added to the solution. Also, the sulfide, which was removed before the Folin-Looney determination was made, corresponds to a further amount of cystine. Such results would suggest that the Folin-Looney reagent responds to some other compound present in the solution. To determine whether or not the optical configuration of a sample of cystine has any effect on its response to the Folin-Looney reagent a series of determinations was run in which pure cystine of  $[\alpha]_D = -215.0^\circ$  was compared with pure racemic cystine. No difference in readings could be detected. Changes in configuration cannot furnish the explanation for these variations.

It is to be noted that the chief result of the presence of the lead





[illegible]

is a rapid increase in the speed of removal of cystine and cysteine. The rate of formation of the latter seems to be more a function of the concentration of alkali while with the lead the increase is more rapidly brought to a stop. The greater irregularity of the figures for cystine and cysteine in the early part of the curve with 4.0 M NaOH is probably to be ascribed to greater speed of reaction with small amounts of oxygen accidentally introduced.

To obtain some idea as to the conditions existing in alkaline cystine solutions when enough time had elapsed to approximate a

TABLE VI.

*Results of Preserving Alkaline Cystine Solutions Away from Air for 128 Weeks at Room Temperature.*

2.00 gm. cystine per 100 cc. in:	Ob- served rotation.	Ob- served rotation after 3 days aera- tion.	Cys- tine.	Cys- teine.	H <sub>2</sub> S	[α] <sub>D</sub> of recovered cystine.
	degrees	degrees	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	degrees
4.00 M NaOH.....	-0.06	-0.13	0.6	1.0	0.001	None re- covered.
2.00 " " .....	-0.02	-0.02	1.4	0.6	0.082	" "
1.00 " " .....	-0.02	-0.02	1.0	0.5	0.086	" "
4.00 " NH <sub>4</sub> OH....	-2.58		0.9	0.2	0.034	-202.0
4.00 " NaOH + 10 gm. lead acetate..	0	0	0	0	0.084 gm. SO <sub>2</sub> .	None re- covered.
2.00 M NaOH + 2 gm. lead acetate..	+0.03	0	0.5	0.4	0.095	" "

condition of equilibrium at room temperature, a series of cystine solutions was prepared as indicated below and preserved in Erlenmeyer flasks for a period of 128 weeks. The solutions were prepared under pure nitrogen, overlaid with a heavy layer of paraffin oil, swept with nitrogen, and tightly stoppered. At the end of the 128 week period each flask was opened and determinations were made of cystine, cysteine, sulfide, and of optical activity. The activity was determined on the acidified solution both at once and after aeration for several days and also on any cystine recovered after neutralization and oxidation. The

observed rotation was determined by pipetting a sample of the solution into excess hydrochloric acid of such concentration as to leave an acidity of about 1.0 M and a cystine concentration of 1.0 gm. per 100 cc. if none had been decomposed. Cystine and cysteine determinations were made by the Folin-Looney method and  $\text{H}_2\text{S}$  was determined as described below.

The results (see Table VI) show an almost complete degree of racemization of what cystine remained in all cases except the ammonium hydroxide solution. In this case about 0.6 gm. of cystine was recovered, which was in characteristic hexagonal plate form and had a rotation of  $-202.0^\circ$ . When lead was present

TABLE VII.  
*Results of Preserving Alkaline Cystine Solutions in Contact with Air  
for 125 Weeks.*

Contents of flask.	Observed rotation.	Cystine.	Cysteine.	$\text{SO}_2$
	degrees	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
2.00 gm. cystine per 100 cc. 4.00 M NaOH...	-0.04	0.08	0.08	0.29
0.5 gm. cystine per 100 cc. 4.00 M NaOH...	-0.03	0.05	0	0.05
2.00 gm. cystine per 100 cc. 1.00 M NaOH...	0	Trace.	0	0.04
0.5 gm. cystine per 100 cc. 4.00 M $\text{NH}_4\text{OH}$ ...	-1.6	"	0	0.01
2.00 gm. cystine per 100 cc. 1.00 M NaOH + 2 gm. lead acetate.....	0	0	0	0.15
2.00 gm. cystine per 100 cc. 4.00 M NaOH + 8 gm. lead acetate.....	+0.09	0	0	0.16
0.5 gm. cystine per 100 cc. 4.00 M $\text{NH}_4\text{OH}$ + 2 gm. lead acetate.....	+0.08	0	0	0.004

in excess (4.00 M NaOH + 10.0 gm. of lead acetate) instead of  $\text{H}_2\text{S}$ ,  $\text{SO}_2$  was evolved. The formation of sulfite, in absence of air appears remarkable. In the other flask containing lead the amount of the latter was insufficient to precipitate all the sulfur. In this case,  $\text{H}_2\text{S}$  was evolved as usual.

A similar series was run in which free access of air was permitted. The flasks were partly protected from unduly rapid evaporation and after standing for about the same length of time as before, the evaporated water was replaced and the same determinations made. These results are recorded in Table VII.

In comparing the data in Tables VI and VII it is evident that

although most of the solutions show little or no optical activity when acidified, the Folin-Looney determination indicates that considerable proportions of racemized cystine and cysteine remain when air has been excluded. Excess of air seems to cause nearly complete disappearance of these amounts particularly in the presence of lead. However, in Table VII, the remarkable case of the solution of cystine in  $\text{NH}_4\text{OH}$ , in absence of any lead, shows the presence of a levorotatory compound which evidently does not reduce the Folin-Looney reagent. Even if no racemization and no reduction occurred, the observed rotation of  $-1.6^\circ$  would correspond to a cystine content of 0.195 gm. per 100 cc. A similar situation is indicated in Table VI. The  $\text{NH}_4\text{OH}$  solution of cystine ended with a remarkably high observed rotation as compared with the Folin-Looney results. The data in Table V show the reverse situation: Folin-Looney determinations indicating more cystine than was originally placed in the flask. It is evident that under the present conditions the results of this method cannot be taken as an unequivocal measure of cystine content.

#### *Production of Sulfide.*

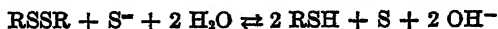
Data on the production of sulfide were included in Tables V to VII in the preceding section. As noted above, when excess plumbite is present the accumulation of sulfide is controlled by the  $\text{Pb}^{++} \times \text{S}^-$  product. In absence of plumbite, sulfide was determined by the following procedure, which consisted in acidifying the sample, blowing the hydrogen sulfide into standard iodine solution, and titrating the excess iodine with standard thiosulfate.

A sample of the alkaline cystine solution was measured into a small gas generating flask provided with a separatory funnel and the necessary inlet and outlet tubes. Before introduction of the sample the flask was supplied with the necessary excess of  $\text{HCl}$  and connected to a train consisting, successively, of a tube containing an asbestos plug, two flasks in series, each containing a measured excess of standard 0.1 N iodine solution with an added excess of  $\text{KI}$ , and a flask containing a solution of  $\text{KI}$  and starch. The addition of  $\text{KI}$  to each flask reduced the possibility of loss of iodine during the determination while the  $\text{KI}$ -starch solution in the last flask served to catch any small amounts of iodine carried over and to indicate any such condition by its color. This solution

was then added to the iodine solution in the other two flasks when the final titration of excess iodine was made. It thus furnished the necessary indicator and demonstrated any loss of iodine. At the end of a determination the color of the KI-starch solution was never darker than a faint blue which required but a fraction of a drop of standard thiosulfate to discharge. The plug of asbestos served to prevent entrainment from the first flask. This plug cannot be made of glass wool. Lead glass, commonly used in glass wool manufacture, reacts with  $\text{H}_2\text{S}$  turning dark and removes an appreciable fraction of the small amount concerned. The use of glass wool always gave erratic results. Pure nitrogen was used to sweep the train during the determination. All air was thoroughly swept out before the introduction of the sample. Experiments on sodium sulfide solutions demonstrated that 4 hours sweeping was sufficient to remove at least 95 per cent of the  $\text{H}_2\text{S}$  present.

The data in Table V show that the production of sulfide in absence of lead is highly influenced by the concentration of alkali. The rapid rise and fall of the sulfide concentration in 4.0 M NaOH is not paralleled in any other solution within the length of time employed (see also Table VI). No other of these reactions shows a speed so directly influenced by the concentration of the alkali.

The amount of sulfide determined at any given time is not to be regarded as the total formed up to that time. The equilibrium



which is responsible for part of the production of cysteine changes part of the sulfide to polysulfide and is undoubtedly responsible for the precipitation of free sulfur when these solutions are acidified.

The production of sulfur dioxide instead of hydrogen sulfide in all cases of atmospheric exposure (see Table VII) is probably of no significance as regards the chemistry of cystine decomposition. In the presence of air and strong alkali the formation of sulfite from sulfide or polysulfide is a common occurrence. Some further oxidation to sulfate was indicated by qualitative tests and various other intermediate oxidation products of sulfur were no doubt present (thiosulfate, *etc.*).

*Production of Ammonia.*

The fact that certain amino acids, including cystine, evolve ammonia on being heated with sodium hydroxide solution has been known for some years. Van Slyke (9) noted that the amount of ammonia derived from boiling a sample of cystine in alkali under certain specified conditions was about 18 per cent of the total when the boiling was done in glass and was raised to over 69 per cent when the same reaction was carried on in a copper flask. More recently Plimmer and Lowndes (10) in investigating the estimation of cystine in the modified Van Slyke method determined the percentage deamination of both pure cystine and "hydrolyzed" (racemic) cystine and found a slightly greater loss of ammonia with the racemic sample.

It appeared worth while to determine if any relationship existed between ammonia production and the other reactions described above. An experiment at room temperature showed plainly the marked influence of plumbite. 1.5 gm. of cystine ( $[\alpha]_D = -207.0^\circ$ ) were dissolved in 100 cc. of 1.00 M NaOH, placed in an aerating flask, and slowly swept with pure nitrogen for several days at room temperature into a flask containing standard acid. At intervals the excess acid was titrated and replaced with a fresh supply. The same experiment was duplicated with the addition of 1.7 gm. of lead acetate to the cystine solution. Table VIII contains the results.

Other experiments yielded comparable figures. Conditions being equal, the effect of the lead was to more than double the rate of ammonia production.

A much more elaborate series of experiments was carried out at  $100^\circ$ . The alkaline cystine solutions were not boiled but were placed in a steam-jacketed container and swept with pure nitrogen at a standardized rate of speed for 5 hours. This procedure gave easily duplicable results and was obviously much preferable to direct boiling of the solution.

To control the effect of variation in the flow of nitrogen, two determinations were carried on simultaneously, in series with each other as regards the nitrogen stream and each day the two consisted of a duplicate of the determination of the previous day plus a new one. Thus duplicates were always run on different days in series with different determinations. In all cases 0.2500 gm. of

cystine was dissolved in 50.0 cc. of the alkali used and any heavy metal salt desired was added. Unless otherwise specified *c. p.* *l*-cystine ( $[\alpha]_D = -207^\circ$ ) was used. The ammonia was swept, as usual, into excess acid and titrated with alizarin. Table IX shows the results obtained.

The data in Table IX show the rather remarkable result that the production of ammonia is *inversely* proportional to the concentration of alkali from 4.00 *M* to about 0.2 *M* NaOH. From 0.2 *M* to 0.001 *M* solutions the speed of ammonia production drops sharply. In the presence of an amount of lead equivalent to the sulfur present the rate of ammonia evolution is more than tripled. This effect is not the result of catalysis by the lead sulfide formed as

TABLE VIII.  
*Deamination of Cystine at Room Temperature.*

Time.	Percentage deamination.	
	Cystine alone.	Cystine + plumbite.
<i>days</i>		
3	2.3	9.2
8	8.1	
10		28.6
13	13.1	
17		41.1
21	20.6	
22		49.1

it was found in other experiments that addition of freshly precipitated lead sulfide to the solution had no effect on the production of ammonia. Moreover, the action is probably not catalytic in nature at all since reduction of the lead to 10 per cent of the theoretical gave a result only slightly higher than that obtained when no lead was present, while doubling the theoretical amount of lead raised the percentage decomposition to 74 per cent.

The figures obtained from copper show a much less marked effect from copper sulfate and no effect at all from the presence of metallic copper in the flask. The effect of ferric chloride was also very slight.

It was presumed that the results with copper and iron were the result of a less effective concentration of these metals since the



TABLE IX.  
*Deaminization of Cystine at 100°.*

Concentration of NaOH.	Heavy metal salts added.	Optical activity of cystine $[\alpha]_D$ .	Percent-age deaminization.
<i>M</i>		<i>degrees</i>	
4.00			10.5
			11.2
2.00			18.1
			17.5
			17.8
			17.9
1.00			21.9
			22.1
0.2181			23.8
			23.5
0.100			21.4
			21.9
0.050			19.9
			18.5
0.010			11.7
			10.4
0.001			3.1
			1.3
2.00	0.079 gm. lead acetate (10 per cent theory).		20.5
			19.8
2.00	0.79 " " " (theory).		61.5
			59.6
2.00	1.6 " " "		74.0
			74.4
2.00	0.3 " copper sulfate.		23.3
2.00	0.52 " " " (theory).		50.2
2.00	0.4 " metallic copper.		17.7
			16.7
2.00	0.375 " ferric chloride (theory).		18.7
			18.0
2.00	0.75 " " "		18.5
			18.1
2.00	0.382 " zinc acetate (theory).		19.3
2.00	0.26 " disodium arsenate.		18.4
			20.0
2.00		-148.7	18.1
			18.1
2.00		0	13.3
			18.2
2.00		+9.0	18.4
			18.0
2.00	0.79 " lead acetate (theory).	+9.0	59.5
			59.6

hydroxides of both were precipitated and that of the copper partly dehydrated. Therefore, zinc was selected as an amphoteric metal with a moderately insoluble sulfide for comparison with lead. The results, however, showed practically no increase over the controls.

On the hypothesis that more complete removal of sulfide ion was necessary than was afforded by zinc, a metal was selected which would effect more complete removal. Arsenic sulfide is not only very slightly ionized but it unites with excess alkali to form soluble and very stable sulf-arsenates. Disodium arsenate in proportion equivalent to the sulfur was used but with negative results. It must be concluded that this very marked effect of lead is of highly specific nature.

In order to determine whether optical configuration had any influence on speed of deaminization a series of cystines was used with specific rotations varying from  $-207^{\circ}$  to  $+9.0^{\circ}$ . The results indicated that optical configuration had no effect, either in the presence or the absence of plumbite.

One of the most outstanding features of the decomposition of cystine in alkali is the marked and specific effect of lead on the various reactions involved. This is particularly true of the rate of loss of cystine and the rate of deaminization. Although the initial formation of cysteine seems to be very little affected by the lead, the latter causes its decomposition to set in more rapidly than would otherwise be the case. This specificity of lead in cystine chemistry leads to interesting speculations as to any possible connection with its very specific properties as a poison. The evident independence of the various reactions studied emphasizes the complexity of the alkaline decomposition of cystine while the ease with which the production of sulfide can be varied by the conditions employed shows the futility of the old classification of "easily split sulfur" unless conditions are very definitely controlled and described.

#### SUMMARY.

The decomposition of cystine in alkaline solution has been studied with reference to the rate of some of the reactions involved. There has been determined, chiefly at room temperature, the rate of formation of sulfide and of cysteine and the rate of loss of cystine. The effect on these reactions of variation in concentra-

tion of alkali and of the presence or absence of lead has also been determined. The presence of lead greatly accelerates the decomposition of both cystine and cysteine.

The potentials of sodium plumbite solution with excess sodium hydroxide against the lead electrode were determined and from these was calculated the probable range of  $Pb^{++}$  concentration in such solutions.

The speed of racemization of cystine in alkali was measured and found to be much greater than the speed of decomposition.

The rate of deaminization was measured under a variety of conditions. This rate is greatly increased by the presence of amounts of lead sufficient to precipitate all the sulfur as  $PbS$ . Copper salts have a much smaller effect and metallic copper none while zinc, iron, and arsenic salts were practically without influence. The specific effect of lead here demonstrated corresponds to its strong influence on the rate of loss of cystine.

The optical configuration of the cystine is without influence on the rate of loss of ammonia.

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## A COLORIMETRIC METHOD FOR THE DETERMINATION OF LIPOIDAL PHOSPHORUS IN BLOOD.

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The lipoids are extracted from oxalated blood with an alcohol-ether mixture and after the solvent is evaporated on the water bath, the organic matter is destroyed with concentrated sulfuric acid and hydrogen peroxide. The excess acid is neutralized with ammonia and reacidified with acetic acid. The phosphate is then precipitated with uranium acetate and determined colorimetrically by a procedure recently described in this *Journal* (1).

### *Procedure.*

In a 10 cc. volumetric flask place 8 cc. of a mixture of redistilled ethyl alcohol 3 parts and ethyl ether 1 part. While rotating the flask introduce into the mixture 1 cc. of well mixed oxalated blood slowly, drop by drop, so as to prevent the formation of large clumps. Immerse the flask in a large beaker of boiling water with frequent and vigorous shaking until the liquid in the flask just begins to boil. Cool to room temperature by immersing the flask in cold water, and add alcohol-ether mixture to the 10 cc. mark. Filter through an ashless filter paper, covering the funnel with a watch-glass to prevent evaporation.

Introduce 5 cc. of the filtrate (equivalent to 0.5 cc. of blood) into a 15 cc. Pyrex centrifuge tube graduated at 10 cc. Immerse the tube in a beaker of boiling water and evaporate to dryness. Add 0.3 cc. of concentrated  $H_2SO_4$  and heat very gently over a small flame of a micro burner until frothing ceases and an abundant supply of  $SO_2$  fumes is produced. Remove the tube from the flame and by holding it in an almost horizontal position rotate it to bring the acid in contact with the adherent lipoidal material on the walls of the tube. Rotate until the walls are completely covered

with the acid and heat again gently for another minute. Let cool and add 5 drops of 30 per cent hydrogen peroxide until the liquid becomes clear but of a deep red color, and again rotate the tube to moisten the walls as before. Boil again until  $\text{SO}_3$  fumes begin to appear. The liquid should now be perfectly clear and colorless. If a yellow tinge should still persist, add another drop of  $\text{H}_2\text{O}_2$  and again heat until the  $\text{SO}_3$  fumes begin to come off. Allow to cool and add 5 cc. of water.

¶ Into a similar test-tube place 5 cc. of standard phosphate solution.

¶ Add to each tube 1 drop of brom-thymol blue indicator solution, and neutralize with dilute ammonia (1 part of 28 per cent ammonia and 4 parts of water) until the solutions just turn blue, and add

TABLE I.

Tube No.	Ammonium sulfate added (50%) cc.	Phosphorus.	Colorimetric readings. Tube 1 used as standard set at 20 mm.
		mg.	mm.
1	None.	0.05	19.9
2	0.1	0.05	20.3
3	0.2	0.05	20.2
4	0.5	0.05	19.5
5	1.0	0.05	20.5
6	2.0	0.05	20.1
7	3.0	0.05	19.7

0.3 cc. more of ammonia. Make just acid with 5 per cent acetic acid; add 1 cc. of uranium acetate solution and let stand for a minute. Add 2 cc. of 95 per cent alcohol, shake well, and let stand for 15 minutes. Centrifuge for about 5 minutes at high speed and decant the supernatant fluid, removing the last drop by touching the mouth of the tube against a clean filter paper. Add 5 cc. of 20 per cent alcohol and with a fine glass rod break up the precipitate and add 5 cc. more of alcohol, washing down the rod. Centrifuge again and wash once more with 20 per cent alcohol. Pour off the final wash fluid as completely as possible and dissolve the precipitate in 1 cc. of 20 per cent trichloroacetic acid. Add 5 cc. of water and 1 cc. of a 10 per cent solution of potassium ferrocyanide, and fill up with water to the 10 cc. mark. Mix well and

after 2 minutes compare in the colorimeter, setting the standard at 20 mm.

*Calculation of Results.*

$$\frac{S}{R} \times 10 = \text{mg. phosphorus in 100 cc. blood.}$$

$S$  = reading of standard in mm.

$R$  = " " unknown " "

DISCUSSION.

Some details of the method, as well as the preparation of reagents, are omitted from this paper, as they have already been described in the previous paper (1).

The choice of a proper solvent for the extraction of lipoids is of great importance. Chloroform and ether do not extract completely all the lipid material; they also present the disadvantage that the tissue must first be dried before being subjected to extraction by these solvents. Kumagawa and Suto (2) found that hot alcohol gave the largest yield of substances soluble in ether; and as pointed out by Bloor (3), it has the advantages in that it penetrates the tissues readily, and can be applied directly to the moist tissue without previous drying. Various solvents have been tried and that of Bloor (4) with alcohol-ether, was found to give the best results.

While it is well known that the phosphorus content of an alcohol-ether extract is not a true measure of the lipoidal content, since other substances containing phosphorus, not lipid, are extracted along with the lipoids, yet this is the only means available, since isolation of lipoids in a pure state is a matter of great difficulty, and utterly impossible in small amounts of tissue.

Another matter of importance is the means used for the destruction of the organic matter in order to liberate the phosphorus. Bloor (4) digests the organic matter with sulfuric and nitric acids. Roe, Irish, and Boyd (5) use sulfuric and nitric acids and complete the digestion with  $\text{H}_2\text{O}_2$ . Baumann (6) uses sulfuric acid only and  $\text{H}_2\text{O}_2$ , so do Koch and McMeekin (7), in the digestion of the Folin-Wu filtrate for the determination of non-protein nitrogen in blood. I have found the sulfuric-nitric acid mixture not as satisfactory in the digestion of lipoidal material as the sulfuric acid alone with the  $\text{H}_2\text{O}_2$ . It was found that 0.1 cc. of concen-

trated  $\text{H}_2\text{SO}_4$  is sufficient to destroy completely all the lipoidal extract from 0.5 cc. of blood. However, 0.3 cc. is used in the method because it shortens greatly the heating time and because this amount of fluid covers more completely the sides of the tube during the process of rotation. This process of wetting the sides of the tube with the acid is very essential, since during the process of evaporation of the alcohol-ether mixture a considerable amount of lipoidal matter sticks to the walls of the tube, and would otherwise escape digestion, thus incurring a loss of phosphorus. It was pointed out by Baumann (8) that on prolonged heating at a high temperature a loss of phosphorus may occur. However, Roe, Irish, and Boyd (5) found that no loss occurs at a temperature of  $200^\circ$ . No loss of phosphorus occurs in the present method. This was checked with added known amounts of phosphate. The whole process of digestion is done very rapidly, in about 2 minutes, and the heating is done very gently over a small flame, so that no superheating occurs.

The next question of importance was to find out whether the ammonium sulfate produced during the neutralization of the sulfuric acid with ammonia does not interfere with the precipitation of uranium phosphate. As Table I shows, ammonium sulfate in great concentration does not interfere with the precipitation of uranium phosphate.

#### SUMMARY.

A method is described for the determination of lipoidal phosphorus in small amounts of blood by extracting the lipoidal material with alcohol and ether. The alcohol-ether is evaporated and the organic matter is destroyed with concentrated  $\text{H}_2\text{SO}_4$  and  $\text{H}_2\text{O}_2$ . The phosphorus is then precipitated as uranium phosphate and determined colorimetrically.

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## OBSERVATIONS BEARING ON THE DETERMINATION OF VITAMIN A.

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About 2 years ago the Protein Laboratory, in cooperation with the Drug Control Laboratory of the Food, Drug and Insecticide Administration, began an investigation of the vitamin content of cod liver oils and products made therefrom. In vitamin A studies we were confronted with certain fundamental considerations of technique.

The United States Pharmacopœia X contains a method for vitamin A assay of cod liver oil. As related by Sherman (1) the technique is essentially that developed by Sherman and Munsell (2), and is based upon the earlier work of Drummond and his associates.

The work of Steenbock and Nelson (3) showed that cessation of growth of rats on a diet deficient in vitamins A and D may be owing to a lack of either one of these factors depending upon the relative amounts stored by the animal before it is put upon the deficient diet. In the Pharmacopœia method proper weight is not given to the growth-promoting influence of vitamin D. Accordingly, under certain conditions the cessation of growth owing to a depletion of the store of this vitamin may be taken as a criterion of the depletion of the animal's store of vitamin A. A previous attempt by one of us to use the Pharmacopœia stock diet met with unsatisfactory results. Within a period of 3 months the condition of the animals indicated nutritive failure. They lost weight, their coats became rough, and reproduction was very poor. By modification of the diet most of the animals were quickly restored to normal condition.

In some of our preliminary work we used a stock diet similar



to that used by Steenbock (5), differing principally in that it contained whole milk powder and no fresh milk. It had the following percentage composition: whole milk powder, 15; yellow corn, 64; commercial casein, 5; linseed oil meal, 13; alfalfa meal, 2; calcium carbonate, 0.5; and sodium chloride, 0.5. This diet proved to be too low in vitamin A for satisfactory results. Many of the females had transient ophthalmia. Reproduction was not satisfactory, and some of the young developed ophthalmia as early as 23 days after being put on a vitamin A-deficient diet, even though they had reached a weight of more than 40 gm. 21 days from birth. The addition of 5 per cent of butter fat to the diet improved it greatly. The corn used was excellent in quality. The alfalfa meal was of poor quality and may not have furnished much vitamin A, but it was surprising that the amount of milk in the diet did not compensate for this deficiency. The milk powder was obtained in 50 pound lots, this quantity being used up in a period of 3 weeks. Possibly the manner in which this material had been handled had been injurious to its vitamin A. We took these observations into consideration in studying the limitations of the Pharmacopœia vitamin A assay of cod liver oil.

We decided to test further the possibility of using the cure of ophthalmia as a quantitative method for vitamin A determination and to study the relationship between such cures and growth response in the animals.

#### EXPERIMENTAL.

##### *Application of the United States Pharmacopœia Method.*

Ten stock female albino rats, primiparas, were divided into two equal groups, Groups A and B, mated, and given the stock diet prescribed by the Pharmacopœia: whole wheat, 33 per cent; whole milk powder, 66 per cent; and sodium chloride, 1 per cent. The males in the two groups were interchanged daily. The milk powder in the diet of Group A was taken from 50 pound tins used for our other stock diets, and for Group B the same brand of milk was taken from 1 pound vacuum cans. Six litters of rats were raised, two from Group A and four from Group B. Two of these litters consisted of six rats each, and the remaining litters were reduced to that number when the young were 7 days old.

We had intended to place all the young rats on a vitamin A-deficient diet at the age and weight prescribed in the Pharmacopoeia method, "not less than twenty-five days and not more than twenty-nine days old, and weighing not less than 35 Gm. and not more than 45 Gm.," but our animals were too large to come within those limits. One litter from Group A (Lot 165) and one litter from Group B (Lot 164) were started on a vitamin A-free diet at 21 days of age. The average weights of these animals were 47 and 44 gm. One litter from each of the foregoing groups (Lot 166 and Lot 167) was started at 25 days of age, the animals averaging 53 and 49 gm., respectively. The two remaining litters from Group B were started at 28 and 29 days and averaged 52 and 61 gm., respectively.

The vitamin A-free diet used in all the experiments described in this paper had the following composition:

	<i>per cent</i>
Casein.....	18
Salt mixture.....	4
Agar.....	2
Yeast.....	8
Dextrin.....	67
Activated peanut oil.....	1

In those experiments in which both vitamins A and D were omitted, the activated peanut oil was replaced by an equal weight of dextrin.

The casein was a commercial product extracted ten times with hot 95 per cent alcohol. Casein in small cloth bags was covered with alcohol and kept at a temperature of 60 to 75° for 7 hours in a specially constructed extractor. The alcohol was then drained off and the casein permitted to drain overnight. Fresh alcohol was then added and the extraction repeated. From 30 to 40 pounds of casein were extracted at one time, and from 20 to 25 liters of alcohol were used for each extraction. The extracted casein was freed from alcohol in a steam-heated drier and then ground. The salt mixture was Osborne and Mendel's Salt IV (4). When used as a sole source of vitamin B, and fed at a level of 0.6 gm. per rat per day, the yeast permitted growth at the maximum rate. Dextrin was prepared from corn-starch by autoclaving the moistened starch for 2 hours at 15 pounds pressure, drying in a current of air

at 50–55°, and then grinding. The peanut oil carried 10 per cent of crude cholesterol which had been irradiated with ultra-violet light to about its maximum antirachitic activity. Besides serving as a vehicle to permit uniform distribution of the activated cholesterol, the peanut oil imparts desirable physical characteristics to the diet. It prevents the settling of finer particles and also prevents the inhalation of dustlike particles which may be particularly irritating to mucous membranes of the respiratory tract which are affected by vitamin A deficiency.

The rats were kept in individual cages provided with false screen bottoms to prevent access to excreta. Each litter was divided into two groups of three rats each. One group received the vitamin A-free diet and the other the vitamin A- and vitamin D-free diet. The rats which received vitamin D grew at practically a normal rate until sore eyes became evident, whereas those which received no vitamin D ceased to grow or grew very little after being on the diet 2 weeks. The average maximum weight of the rats on the vitamin A-free diet before showing ophthalmia was 116 gm., ranging from 101 to 135 gm. The corresponding average maximum weight for the vitamin A- and vitamin D-free diet rats was 79 gm., with a range of 68 to 100 gm. The average gain for all the rats receiving vitamin D previous to vitamin A additions was 37 gm. more than the rats which received no vitamin D. In all cases where the rats received vitamin D ophthalmia developed nearly simultaneously in each litter. In only one litter, Lot 165, did sore eyes become evident as early in the rats which received no vitamin D as in those which received that component. The delay in most cases was a few days, but in three instances unmistakable ophthalmia did not develop until from 44 to 50 days.

Four of the litters were given butter fat as a source of vitamin A when ophthalmia had developed. The butter fat additions to the diet were made when the condition of the eyes could be easily recognized as abnormal, but before suppurative conditions had become established. There was marked redness and some swelling of the lids, and in many cases bleeding or slight lacerations due to scratching. Depilation of the lids, quite characteristic of hooded rats in the early stages of xerophthalmia, is not so readily recognized in the albinos. Lots 164 and 165 received 100 mg. of butter fat each daily, and Lots 166 and 167 received 200 mg. The butter

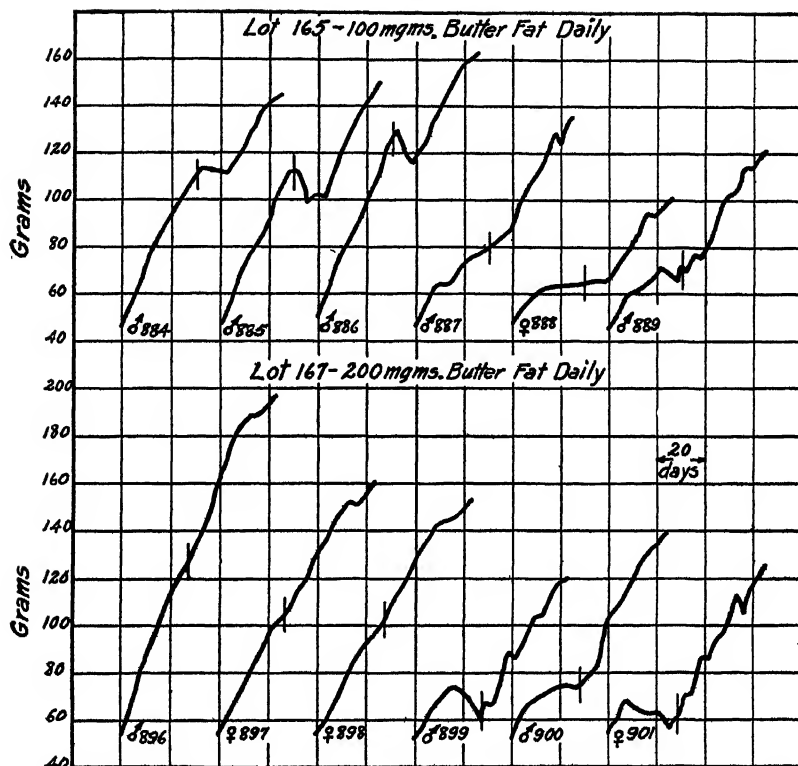


CHART I. The curves show the rate of growth of rats on a vitamin A-deficient diet with and without vitamin D, and the extent of growth induced in 35 days of butter fat feeding after ophthalmia had been produced. The beginning of butter fat feeding is indicated by a short vertical line intersecting the growth curve. The animals in Lot 165 were from one litter and Lot 167 another. The first three rats in each litter received an adequate amount of vitamin D from irradiated cholesterol throughout the experiment. The last three rats in each litter received no vitamin D except that contained in the butter fat. Previous to the butter fat addition the rats which received vitamin D grew much more rapidly than those which did not, but after the butter fat addition the average increase in weight was slightly greater in the rats which had received no vitamin D.

fat was prepared from unsalted butter. It was melted and filtered to remove proteins and water, and stored at a freezing temperature. Each day a portion was brought to a temperature of 60°, and the desired amounts were transferred with a graduated pipette to small glass dishes, and fed.

The growth curves of Lots 165 and 167 shown in Chart I are typical of the performance of the animals in the other two lots. In no case was there a cure of ophthalmia on the 100 mg. level of butter fat up to the end of 35 days, when the experiment was terminated. In general, there was a slow improvement during the first 2 or 3 weeks with little, if any, change after that. On the 200 mg. level there was an early improvement in the eye condition of all the animals and with one exception all the animals became practically free from symptoms of vitamin A deficiency in from 10 to 22 days. In nearly all cases there was a slight recurrence of redness of the lids, usually in one eye, which was easily discernible for intervals of a few days, then disappeared. One rat was never free from ophthalmia.

The average increase in weight for six rats on the vitamin A-free diet which received 100 mg. of butter fat daily was 42 gm., and for those which received 200 mg. of butter fat it was 63 gm. The average weight increase of six rats on the vitamin A- and vitamin D-free diet which received 100 mg. of butter fat was 50 gm., and for those which received 200 mg. it was 60 gm.

The difference in the time of onset of ophthalmia in Groups A and B was not marked, although the young from Group A showed these symptoms an average of 5 days earlier than did rats started from Group B at the same age and nearly the same weight. The data obtained were too limited to furnish conclusions as to the relative merits of using whole milk powder from small evacuated containers or from large containers with no special precautions taken to preserve vitamin A.

#### *Cure of Ophthalmia and Growth as an Index of Vitamin A Potency.*

In testing medicinal cod liver oils for the vitamin A potency we have used the cure of ophthalmia as a criterion and at the same time obtained information on the amount of growth induced during the curative period. The rats used in these experiments were taken from our stock colony which received a diet essentially the

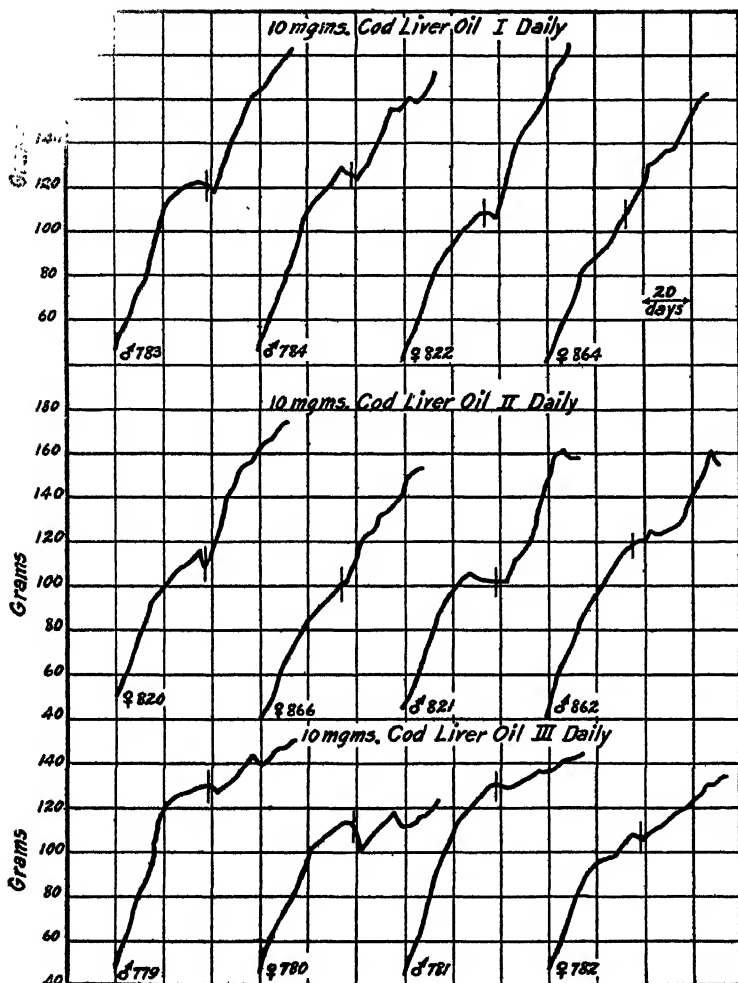


CHART II. These growth curves are typical of the results obtained in testing cod liver oils by the technique described in the text. The vertical lines intersecting the growth curves mark the beginning of cod liver oil feeding. The curative test was begun when ophthalmia was definitely established. The weights of the animals when the curative tests were begun varied from 100 to 130 gm. Only one animal remained stationary or declined in weight for a period of 7 days previous to the time cod liver oil feeding was begun. The average weight increase during the 35 day test period on Cod Liver Oil I was 60 gm.; Cod Liver Oil II, 52 gm.; and Cod Liver Oil III, 19 gm.

same as that used by Steenbock (5). In place of whole milk the animals received whole milk powder incorporated in the diet, and some received butter or carrots in addition. Rats 21 to 23 days old and weighing 40 to 45 gm. were given the vitamin A-free diet previously described until ophthalmia developed. During this preparatory period the rats, in groups of two or three, were kept in cylindrical wire cages 8 inches high and 11 inches in diameter, having raised bottoms of galvanized wire with three meshes to the inch. When the rats showed sore eyes which could be easily recognized, red or slightly swollen and frequently bleeding lids, they were put in similar individual cages and given cod liver oil. Such rats have ranged from 100 to 145 gm. in weight. The cod liver oil was mixed with peanut oil so that the amount to be fed each day would be contained in 0.2 cc. of the mixture. The oil was placed before the animals in small glass dishes. The curative period was limited to 35 days. Four rats from four different litters were used in each test.

The growth curves resulting from one test on each of three cod liver oils are given in Chart II. These results were selected from more than 50 tests carried out on twenty-six cod liver oils as being typical of the performance of our animals. They also represent the range of variation in vitamin A of about 90 per cent of the oils examined. Cod Liver Oil III is the lowest in vitamin A examined to date, and two oils have been found to be distinctly richer in vitamin A than Cod Liver Oil I. There were three rapid cures of ophthalmia on Cod Liver Oil I. Rat 864, whose eyes showed a slightly more advanced stage of ophthalmia when changed, did not show complete absence of the disease during the test period. On Cod Liver Oil II three rats were completely cured of ophthalmia, but not as rapidly as on Cod Liver Oil I. Rat 862 was not completely cured. The condition of the eyes of the rats on Cod Liver Oil III remained practically the same during the test period.

The performance of control rats kept on the vitamin A-free diet simultaneously with the cod liver oil tests is as follows: An average weight of 100 gm. was attained in 20 days, a gain of nearly 3 gm. per day. The growth rate then gradually declined, and a maximum of 120 to 125 gm. was reached between the 40th and 45th day. At this point the range in weight was from 100 to 150 gm., with a marked increase in weights of males over females. Deaths

occurred as early as the 45th day, and one animal lived 85 days. The length of survival was extremely variable, even with animals from the same litter. Symptoms of ophthalmia could usually be detected between the 35th to the 40th day, but there were variations ranging from 30 to 46 days.

#### DISCUSSION.

The results which we present with respect to the application of the United States Pharmacopœia vitamin A assay of cod liver oil are not to be considered typical of the results to be obtained by this method. They are presented to point out certain defects in the Pharmacopœia assay which the producers of cod liver oil, who are principally interested in this method, have undoubtedly encountered. The Pharmacopœia assay provides in part:

"The rats shall begin receiving the Cod Liver Oil to be tested after not less than seven days of stationary or declining weight, and from the time of feeding the test oil, they shall be kept in separate cages."

"The test period shall continue for thirty-five days and the potency of the oil shall be judged from the rat or rats showing a gain on the thirty-fifth day of between 10 and 20 Gm. over the weight at the beginning of the test, and the cure of the induced symptoms of vitamin A starvation."

It is obvious from Chart I that the stock diet was so low in vitamin D that storage in the young was very low. If the animals in Lot 167 had been changed to the material tested after 7 days of stationary or declining weight, Rat 901 would have been changed after 14 days; Rat 899, 21 days; and Rat 900, 28 days, on the vitamin A- and vitamin D-free diet. The growth of the rats on the vitamin A-free diet shows the fallacy of depending on the growth rate to determine when stored vitamin A is depleted in the animal, if vitamin D is not provided in adequate amounts.

If the Pharmacopœia method is modified so that vitamin D is supplied to the test animals, our experience indicates that other details of that method cannot be followed to advantage. There may be retardation of the growth rate before symptoms of ophthalmia become apparent. If the curative test is not begun until the animal has been at a stationary or declining weight for a period of 7 days, the test must be conducted on an animal that may have reached a critical condition, and that condition will not be greatly improved if the level of vitamin A fed permits but little



growth. (In our method of feeding the oil, results are further complicated by unsatisfactory oil consumption on such low levels.) Furthermore, we do not get uniform cures of mild cases of ophthalmia during the test period unless the animals receive sufficient vitamin A to permit growth at approximately the maximum rate. For that reason the growth requirements in the Pharmacopœia become meaningless if the induced symptoms of vitamin A starvation are cured.

Ophthalmia has occurred in practically 100 per cent of the animals used in our vitamin A tests. Over a period of 2 years only two rats failed to develop detectable symptoms of sore eyes on vitamin A-deficient diets. One of these animals died after being on the experimental diet 35 days, a few days before its litter mates showed ophthalmia. The other rat died at 52 days, 6 days after the other animals in the litter had been put on curative tests. Aside from the fact that the animals we used must have had a comparatively low storage of vitamin A, we attribute our success in developing ophthalmia to the fact that we used only vigorous animals, that grew at a rapid rate on the vitamin A-free diet.

Just what is intended by the statement in the Pharmacopœia that, "The antirachitic requirements of the breeders shall be assured." is not clear to the writers. If it means that reproduction and subsequent growth of the lactating young shall not be influenced by a lack of vitamin D, which seems the most plausible interpretation, then it is quite probable that no vitamin D other than that contained in the prescribed stock diet is necessary. We are not familiar with any studies in which the Pharmacopœia technique has been applied where an attempt has been made to provide the mothers with sufficient vitamin D to enable the young to acquire enough of that vitamin so that the amount of growth on a vitamin A- and vitamin D-free diet will be dependent only on the vitamin A storage. If this can be done successfully, the first symptoms of vitamin A deficiency will be the same as when the test diet is deficient only in vitamin A, and from 10 to 20 mg. per day of most cod liver oils will be required to meet the Pharmacopœia definition for a unit. If the United States Pharmacopœia method had contained a statement to the effect that adequate vitamin D must be provided the test animals at all times, it is very doubtful if cod liver oils would be marketed at the present time with claims

which could be justified of containing 500 or more U. S. P. units of vitamin A.

We believe that the technique we have used in testing cod liver oil will give more trustworthy results than are generally obtained by the Pharmacopoeia technique. The rate of growth during the test period is a more tangible manifestation of vitamin A potency than cure of ophthalmia, but data should be obtained on both. Some cases of ophthalmia apparently cured will recur a few days later and again disappear. It therefore becomes difficult to define a cure of ophthalmia. Relative results are not so difficult to obtain, but a definite standard which an oil must meet is not easily arrived at.

Vitamin A can be determined quantitatively by a technique which will eliminate most of the criticisms directed at the Pharmacopoeia method. Data have been obtained in this laboratory which show that by using suitable controls one dose of cod liver oil can be fed and effect a delay in onset of symptoms of vitamin A deficiency in proportion to the amount of vitamin A in that oil. We prefer to withhold further discussion of these results until the limitations of the method have been better established.

Our results on butter fat feeding are in agreement with those of Sherman (1), in so far as the growth response is concerned. Supplying vitamin D in generous quantities did not markedly affect the rate of growth during the test period. Vitamin D tests on the butter used showed that it had less than  $\frac{1}{10}$  the vitamin D potency of a good grade of cod liver oil. The vitamin D requirement for growth of a rat at a rapid rate is apparently very low if the other constituents of the diet are supplied in proper proportions.

In comparing the responses we got in feeding butter fat with cod liver oil, it appears that the butter fat had about one-fifteenth the vitamin A potency of a good grade of cod liver oil. These results are in harmony with those of Jones, Steenbock, and Nelson (6). The exact history of the butter we used is not known. It was obtained during the month of April from the Bureau of Dairying and, although not as deep in color as butter obtained later in the year, could be considered a good grade of butter.

The authors wish to acknowledge the assistance of J. P. Devine and J. G. Wangler of this Division, in carrying out the experimental work described in this paper.

## SUMMARY.

1. Data relating to the determination of vitamin A are presented, and certain defects of the United States Pharmacopœia vitamin A assay of cod liver oil are discussed.

2. The United States Pharmacopœia X method does not eliminate vitamin D as a growth-promoting factor. When this factor is provided ophthalmia usually develops before growth ceases, so that the curative test cannot be carried out on an animal free from disease.

3. In curative tests on animals which have developed incipient ophthalmia permanent cure of ophthalmia does not take place unless the level of vitamin A fed is high enough to permit growth of the rat at or near its maximum rate.

4. In curative tests for vitamin A, although observations on the presence and change of severity of ophthalmia are essential, the growth response seems to offer a more tangible means of judging vitamin A potency.

5. The vitamin A potency of a sample of butter fat was found to be approximately one-fifteenth of that of a good grade of cod liver oil.

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## ON CEREBRONIC ACID. VI.

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(Received for publication, July 31, 1928.)

In a recent paper Klenk<sup>1</sup> has emphatically refuted the accepted view of the structure of cerebronic acid as  $C_{25}H_{50}O_3$ . This statement seems very surprising in the light of the experience of these laboratories. Three different workers, each at a different time, have obtained lignoceric acid on oxidation of cerebronic acid. In the experiments reported by Levene and Taylor<sup>2</sup> the yield of lignoceric acid on oxidation of cerebronic acid was 81 per cent of the theory. A yield of this proportion could not be attributed to an impurity. Furthermore, the lignoceric acid obtained from the cerebronic acid was converted into the corresponding pentacosanic acid, which had the correct molecular weight, and the latter acid again was converted into a hydroxypentacosanic acid, which had the correct molecular weight. It is hard to conceive how Klenk considers his evidence more convincing than that furnished by Levene and West<sup>3</sup> and by Levene and Taylor.<sup>2</sup> However, in view of the very emphatic assertions of Klenk, we concluded to repeat the oxidation of cerebronic acid under more rigorous conditions than in the experiments of Levene and Taylor. Nevertheless, the main product of oxidation was an acid of the composition  $C_{24}H_{48}O_2$ , having the molecular weight of 369 (the theory requiring 368) and a melting point of 79.0–80.5°. Thus we shall adhere to the older view of the composition of cerebronic acid until Klenk shall have furnished more convincing evidence in favor of his theory. We may add, however, that we have some

<sup>1</sup> Klenk, E., *Z. physiol. Chem.*, 1928, clxxiv, 214.

<sup>2</sup> Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1922, lii, 227.

<sup>3</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913–14, xvi, 475.

reason to believe that the acid  $C_{23}H_{40}O_2$  of Klenk was a mixture of two acids, one of  $C_{24}$  and the other of  $C_{22}$ . A pure sample of the acid  $C_{22}H_{40}O_2$  was obtained by us in an experiment in which a larger sample of cerebronic acid was oxidized by the usual method. The cerebronic acid in that experiment had a melting point of  $99.5-100.5^\circ$  and a specific rotation of  $[\alpha]_D = +3.7^\circ$ .

#### EXPERIMENTAL.

*Preparation of Phrenosin.*—Mixed crude cerebrosides which had been kept in the laboratory for several years were purified in the following way. First, they were recrystallized several times from glacial acetic acid and this product was then recrystallized a few times from pyridine. The material was freed from pyridine by extraction with acetone. The dry residue was then recrystallized several times from a solution of equal parts (by volume) of methyl alcohol and of chloroform until further recrystallization no longer affected the specific rotation of the phrenosin. The rotation of this material in pyridine was  $[\alpha]_D^{20} = +5.0^\circ$ .

*Cerebronic Acid.*—The cerebronic acid was obtained either by hydrolysis in an autoclave for 24 hours, or by alcoholysis by means of alcohol containing 8 per cent of sulfuric acid. The acid was purified by conversion into the lead salt and subsequent removal of the lead ion. Often the operation had to be repeated several times. The purified material melted at  $130^\circ$  and gave the following analysis:

4.355 mg. substance: 12.040 mg.  $CO_2$  and 4.970 mg.  $H_2O$ .

0.500 gm. " required 12.55 cc. of 0.1 N NaOH.

$C_{22}H_{40}O_2$ . Calculated. C 75.33, H 12.50, mol. wt. 398.

Found. " 75.39, " 12.76, " " 398.5.

The molecular weight was 398.5 and the optical rotation in pyridine was

$$[\alpha]_D^{25} = \frac{+0.14^\circ \times 100}{1 \times 3.0} = +4.66^\circ.$$

*Oxidation of Cerebronic Acid.*—Of the cerebronic acid just described, 24 gm. were oxidized in acetone solution with potassium permanganate, an excess of permanganate being employed. The oxidation was carried out in three lots, practically in the same

manner as described by Levene and Taylor, with the slight modification that each sample of 8.0 gm. of the acid was oxidized in 1000 cc. of acetone containing 10.0 gm. of potassium permanganate. By extracting the manganese dioxide with hot methyl alcohol the salts of the fatty acids were obtained, which yielded 14.0 gm. of fatty acids. These were fractionated in the manner described in the previous paper. Two fractions were obtained. The lithium salts were converted into the free acids, which were recrystallized from acetone. The larger fraction in this state of purity had the following composition.

4.140 mg. substance: 11.885 mg.  $\text{CO}_2$  and 4.995 mg.  $\text{H}_2\text{O}$ .

$\text{C}_{24}\text{H}_{48}\text{O}_2$ . Calculated. C 78.15, H 13.13.

Found. " 78.28, " 13.50.

The yield was 9.0 gm. It was then further purified by being passed through the lead salt. The acid then had the following composition.

3.775 mg. substance: 10.805 mg.  $\text{CO}_2$  and 4.470 mg.  $\text{H}_2\text{O}$ .

0.3000 gm. " required 8.10 cc. of 0.1 N NaOH.

$\text{C}_{24}\text{H}_{48}\text{O}_2$ . Calculated. C 78.15, H 13.13, mol. wt. 368.

Found. " 78.05, " 13.24, " " 370.

The molecular weight was 370. The acid melted (not absolutely sharply) at  $79-80^\circ$ . It was recrystallized several times from acetone and then from ether. The melting point was raised only slightly,  $79.0-80.5^\circ$ , and the molecular weight remained unchanged.

0.300 gm. substance required 8.13 cc. of 0.1 N NaOH. Mol. wt. 369.

The substance was then esterified. 5 gm. of the acid were taken up in 200 cc. of 98.5 per cent alcohol containing 2.5 gm. of sulfuric acid and refluxed overnight. On cooling, the ester settled out in plates. The ester was taken up in ether, washed free of adhering sulfuric acid, and the ester obtained on evaporation of the ether was recrystallized from alcohol. It melted sharply at  $56-57^\circ$ .

The mother liquor from the first crystallization was concentrated to 100 cc. and in the solution a deposit of glistening plates formed. The yield was 1.3 gm. It was dissolved in ether and freed from sulfuric acid. The residue from the ether was re-

crystallized from alcohol. The substance melted at 79–80°. It had the following composition.

0.0986 gm. substance: 0.2824 gm. CO<sub>2</sub> and 0.1156 gm. H<sub>2</sub>O.  
0.2000 " " required for neutralization 5.45 cc. of 0.1 N NaOH.  
C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>. Calculated. C 78.15, H 13.13, mol. wt. 368.  
Found. " 78.10, " 13.22, " " 367.

Hence this substance was lignoceric acid.

The ester melting at 56–57° was distilled. It distilled at 205–210°, leaving only a small residue. The distillate melted at 56–57°. The ester was converted into the free acid in the usual way. The acid, after recrystallization from benzene, melted at 78–79° and solidified at 76°. It had the following composition.

0.0986 gm. substance: 0.2830 gm. CO<sub>2</sub> and 0.1156 gm. H<sub>2</sub>O.  
0.2000 " " required for neutralization 5.43 cc. 0.1 N NaOH.  
C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>. Calculated. C 78.15, H 13.13, mol. wt. 368.  
Found. " 78.26, " 13.22, " " 368.3.

The lower melting points may be due to the fact that the cerebronic acid was obtained from cerebroside prepared many years ago, or perhaps to slight admixtures of the lower acids to be described in another paper.

# SYNTHESIS OF VITAMIN B IN THE RUMEN OF THE COW.\*

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(Received for publication, September 14, 1923.)

In our studies concerning the vitamin B requirement of calves, one of us produced conclusive evidence that a calf will grow normally to maturity and produce normal offspring on a ration that carries an insufficient amount of the vitamin B complex to support growth and well being in rats (1). In later investigations we determined that vitamin B in milk is not dependent on the presence of this vitamin in the ration of the cow (2). Three cows that were fed for over 2 years, throughout their growth period, on a ration that was decidedly deficient in vitamin B, were used in this study. The evidence obtained in these investigations appeared to indicate that cattle, and possibly all other ruminants, possess the ability to synthesize vitamin B. Damon (3), Kuroya and Hosoya (4), Heller *et al.* (5), Scheunert and Schieblich (6), Sunderlin and Werkman (7), and others have made contributions to our knowledge of the possible synthesis of vitamin B complex by bacteria. The present paper is intended to set forth the results of an investigation which was designed to determine whether the microorganisms present in the rumen of one of our experimental cows were responsible for the synthesis of vitamin B complex.

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## EXPERIMENTAL.

A pure bred Holstein heifer, Penstate Homestead Jessie, No. 924062, was made the subject of this study. She was one of a group of seventeen experimental animals, and had grown to maturity on the experimental ration, which was made up from corn gluten meal, cane sugar, commercial casein, polished rice, corn-starch, pearled hominy, dried sugar beet pulp, cod liver oil, and mineral supplements. This ration was demonstrated, through rat feeding trials (1), to be practically devoid of the vitamin B complex.

A permanent fistula about  $3\frac{1}{2}$  inches in diameter was made in the rumen of the experimental heifer through the left side. This provision afforded an easy means of sampling the rumen contents. A special pack, after the method used by Schalk and Amidon (8), was provided for keeping the fistula tightly closed so that normal conditions within the rumen would be maintained. The heifer continued to thrive (on the ration deficient in vitamin B) and gave every evidence of functioning normally as she had done on the experimental ration, prior to the operation.

Our first step in the investigation consisted in testing out alcoholic extracts from the fermented rumen contents. 12 hours after feeding, portions of about 30 pounds of the wet fermented feed were removed from the rumen and placed in a 5 gallon milk can. The fermented material was incubated for 5 days at a temperature of  $37^{\circ}$ , with occasional stirring of the mixture in order that the bacterial flora might multiply and produce, if possible, the maximum quantity of vitamin B. This procedure was considered preliminary, and precautions were taken to provide an extract sufficiently concentrated to eliminate possible difficulties in feeding technique. A total of 56,530 gm. of the fermented rumen contents, representing four separate samplings, was used in preparing the extract. This represented 6252 gm. of dry matter, the average percentage of dry matter being 11.06 per cent.

The fermented rumen contents were stored in 5 gallon stone jars. 95 per cent ethyl alcohol was added to the jars in liberal amounts because of the large amount of moisture present. It was the aim to provide a solution of approximately 70 per cent alcohol. The jars containing the mixture were allowed to stand for 1 week at room temperature with occasional stirring each day.

The liquid was then filtered off and evaporated to dryness at a temperature of 50°, after the usual practice of preparing vitamin B extracts. 2226 gm. of extract carrying 1200 gm. of dextrin, were so prepared that 1 gm. of this extract represented 25.4 gm. of the original fresh fermented rumen material.

### *Rat Feeding Experiments with Rumen Extract.*

The feeding technique used in this and later feeding trials is essentially that described by Dutcher, Francis, and Combs (9). The animals were placed on experiment when they were 21 days of age, and their average weight was about 40 gm. Males and females were used in equal numbers, and litter mates were distributed throughout the groups. Each rat was kept in an individual cage provided with a screen bottom (10). Growth records were obtained and food intake noted.

The animals refused to eat the ration containing rumen extract for the reason that it carried a decided butyric acid odor. 3 per cent of olive oil incorporated in the ration proved to make it palatable. The ration finally adopted (Ration 103) was made up as follows:

	<i>per cent</i>
Casein.....	18
Salts 185*.....	3
Agar.....	2
Olive oil.....	3
Dextrin.....	24
“      rumen extract.....	50
	<hr/>
	100

\* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxi, 181.

The controls also receive an equivalent of olive oil in their ration. Each rat was given 4 drops of Squibb's cod liver oil daily to insure an adequate supply of vitamins A and D.

The average growth curve for the two groups is shown in Chart I. In the 8 week period the groups receiving the rumen extract made an average gain in weight of 5.2 gm. per week (Curve I).

The growth curves indicate clearly that the rumen extract must have supplied the vitamin B complex since all animals maintained a constant rate of growth throughout the 8 week period. The

controls (Curve II, Chart I) were significantly different although they continued to live (some of them for as long as 10 weeks), but their increases were very slight during the 8 week period.

The evidence obtained in this feeding experiment seemed to indicate quite clearly that vitamin B must have been synthesized by bacteria or other microorganisms. The next step was to make a study of the microflora in the rumen.

### *Bacterial Study of Rumen Microflora.*

The work of Damon (3) on acid-fast bacteria which synthesized the vitamin B complex, suggested that we might be dealing with a similar situation in the rumen of our experimental cow. We had also expected to find wild yeasts. Direct microscopic examination

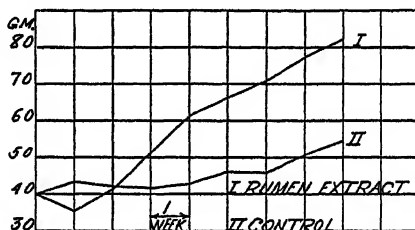


CHART I.

of stained smears of the rumen contents revealed the presence of neither acid-fast bacteria nor yeasts. Samples plated with plain nutrient agar revealed approximately 2,225,000 bacteria per gm. of rumen contents but no yeast colonies were found. What we did find was an almost pure culture of a bacterium that produced a small colony about 1 to 2 mm. in diameter. The colonies developing upon the surface showed a pale lemon-yellow pigment. The subsurface colonies developed no pigment but when transfers were made to agar slants, they proved to be identical with the surface colonies. A search of the literature revealed that our organism had never been described. We have, therefore, designated it *Flavobacterium vitarumen*. Platings on plain nutrient agar of several samples showed the results indicated in Table I. The predominance of this type of colony in our first plates prompted us to make preparations for a test of its ability to synthesize the vitamin B complex.

Plain nutrient agar medium, made from the dehydrated Bacto-Nutrient Agar manufactured by the Digestive Ferments Company, was placed in 32 ounce bottles of the Blake type and sterilized for 20 minutes at 15 pounds pressure. The bottles were placed in a horizontal position in order that the agar would solidify on one side of the bottle and provide the maximum area for growth. The media in these bottles were then inoculated with a water suspension of a pure culture of the organism and incubated for 48 to 72 hours at 37°. The growth, which was not abundant, was then washed from the surface of the media with sterile water and the washings were evaporated to dryness at a temperature of approximately 50°. The amount of growth secured in this manner was less than 1 gm. of dried material per bottle. We were

TABLE I.

Date.	Total No. of bacteria per gm.	Approximate percentage of <i>Flavobacterium vitarumen</i> .
Feb. 21.....	2,225,000	98
" 26.....	8,500,000	98
Mar. 4.....	1,840,000	97
" 17.....	1,850,000	96
June 30.....	26,300,000	95
July 13.....	27,450,000	90
Aug. 1.....	34,500,000	50

able, however, to prepare a sufficient quantity of dried bacterial cells in the form of a meal to make the necessary test for vitamin B in the rat feeding experiments.

*Flavobacterium vitarumen* is rod-shaped, being 0.5 to 1.5 microns  $\times$  0.5 — 3.0 microns in size. It has rounded ends, does not form endospores, generally occurs singly but occasionally in pairs, and is non-motile. It differs from the vast majority of the genus *Flavobacterium* in that it is Gram-positive.

Surface agar colonies are 1 to 4 mm. in diameter, while the imbedded colonies are somewhat smaller. Gelatin colonies are very similar to the agar colonies. Other cultural characteristics are as follows: Agar slant, filiform; chromogenesis, pale lemon-yellow; gelatin stab, no liquefaction; sugar broths, no gas produced; sucrose, dextrose, and maltose broth, acid produced;

lactose, xylose, dulcitol, mannitol, sorbitol, and inositol broth, no acid produced; starch agar, no diastase; cellulose, no fermentation; lead acetate agar, no growth; Endo's medium, no growth.

Litmus milk is fermented with the production of acid. There is a slight reduction of the litmus but no curd is formed.

It does not form a ring of pellicle growth on any of the broth media. The growth clouds the liquid and finally settles. It does not form indole or skatole and nitrates are reduced.

#### *Rat Feeding Experiments with Dried Bacteria.*

The feeding technique was the same as that employed in the previous test on the rumen extract. The dried bacterial meal

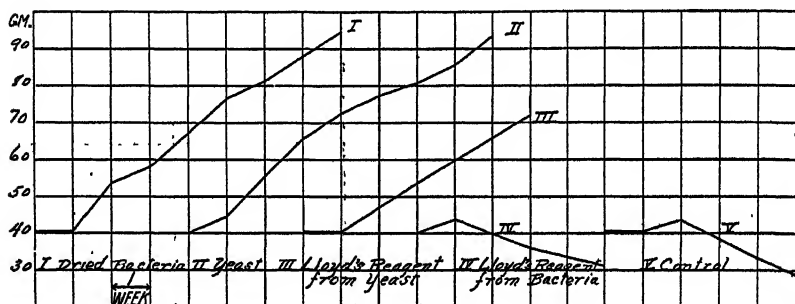


CHART II.

was fed daily in 0.5 gm. doses to each rat as a supplement to the basal ration. In Curve I, Chart II, the result of this feeding test is presented. The curve represents the average growth of ten rats. The average weekly gain per rat was 6.8 gm.

At the same time another group of rats, some of which were litter mates of those represented in Curve I, were fed the basal ration supplemented with 0.5 gm. of Fleischmann's special yeast. In Curve II the average growth of this group is presented. The average weekly gain per rat in this group was 6.4 gm.

There was some question as to whether the media (dehydrated nutrient agar) which were used in growing the large quantities of bacterial cells, did not carry appreciable amounts of the vitamin B complex. To test out this point, a liter of medium made from Bacto-Nutrient Agar was shaken with 10 gm. of Lloyd's reagent.

The mixture was filtered and the filtrate evaporated to dryness. The residue was fed to rats in daily 0.5 gm. doses with the result as presented in Curve IV, Chart II. A similar test was made with autolyzed yeast, prepared as described by Seidell (11). Curve III represents the average growth of this group. A control group was also fed on the basal diet with the results portrayed in Curve V.

#### SUMMARY AND CONCLUSION.

Investigations were conducted on the fermented rumen contents of a Holstein cow, representative of a group of seventeen animals that were grown to maturity on a ration highly deficient in vitamin B complex. Alcoholic extracts of the fermented rumen contents were proved potent in the vitamin B complex through rat feeding trials.

One bacterium of the genus *Flavobacterium* was found about 90 per cent predominant in the rumen microflora. This organism was grown in large quantities on vitamin B-free media and fed to rats to the extent of about (12 per cent) of dried bacterial cells in a synthetic vitamin B-free ration. This test proved the bacterial cells to be highly potent in the vitamin B complex.

The results of this study warrant the conclusion that the vitamin B complex was produced in the rumen of our experimental cow by bacterial fermentation. This result offers a satisfactory explanation as to why cattle, unlike any other species of animal yet studied, have the ability to grow to maturity, to produce normal offspring, and to produce milk of normal dietary composition, on a ration that carries an insufficient amount of vitamin B complex to support growth and well being in rats.

Credit is due Dr. J. F. Shigley, of the Department of Animal Husbandry, for veterinary service in caring for the experimental heifer with the permanent fistula.

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## STUDIES ON THE COMPOSITION OF HUMAN MILK.\*

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(Received for publication, August 6, 1928.)

Numerous reports concerning the composition of human milk and colostrum are to be found in the literature, but most of them are based on only a few cases. The present investigation includes analyses of the milk of 88 normal women for protein, sugar, fat, and in 60 of these cases for ash. Two samples were obtained from each subject, one on the 5th and one on the 9th day post partum, and one or two additional samples were procured from 50 of these subjects at later periods, ranging from 3 to 8 weeks post partum. The average composition of the milk at different periods and the variations observed were studied and compared with the results of others. An attempt was also made to investigate the effects, if any, of different diets on the composition of the early milk. Ordinary hospital diet, high carbohydrate, and high fat diets were used. The infants of the above mothers and others who were fed the same diets but whose milk was not analyzed—130 in all—were weighed daily while in the hospital. 75 of these were brought back to clinic one or more times and were weighed at each visit. An average growth curve has been plotted from these weights.

### *Procedure and Methods.*

The diets used were as follows: Diet 1, ordinary hospital diet; Diet 2, ordinary hospital diet plus 60 gm. of rice daily; Diet 3, ordinary hospital diet plus 60 gm. of tapioca daily; Diet 4, ordinary hospital diet plus 100 gm. of 40 per cent cream daily. Diets 2, 3, and 4 were calculated to have the same caloric value; namely,

\* Preliminary reports of this work have been published by Kleiner and Bell (7) and Kleiner, Tritsch, and Graves (8). The latter article stresses the clinical side of the question.



about 3000 calories. Diet 1 contained about 2600 calories. Each mother was given the specified diet from the time of delivery until she left the hospital. She was encouraged to continue the same diet at home, but since this condition could not be supervised no conclusions have been drawn regarding the effect of diet after the patients left the hospital.

The milk was obtained in every case by an electric breast pump, one breast—the one not used at the previous nursing—being completely drained. On the 5th and 9th days post partum the milk was taken at approximately 9 a.m. Later samples after the patients left the hospital were taken in the afternoons when the mothers returned to the hospital clinic. The babies were weighed at these times also.

In nearly all cases the analysis of the milk was started immediately after the samples were obtained. Occasionally specimens were placed on ice until the next day, but in no case was the analysis postponed more than 24 hours. The complete sample was mixed and the volume and specific gravity were taken. Fat was then determined by the Babcock method, protein by the Kjeldahl method (total nitrogen  $\times 6.37$ ), lactose by the Owen and Gregg (10) modification of the Folin and Wu blood sugar method, and ash by evaporation with concentrated nitric acid and ignition at a low temperature.

### *Results.*

The averages of all samples of milk analyzed are shown in Table I. Since wide variations occurred among the different cases maxima and minima for each period are included. These averages and the weight curves of the infants are plotted in Chart I. An example of the variations in the milk at different periods is given in Table II, which is a record of a typical case on ordinary hospital diet.

In considering the averages of all analyses, the following observations have been made. The protein content of the milk decreased rapidly at first, then more slowly. The fat and sugar increased, first rapidly, then slowly, until a constant level was reached and maintained. Ash decreased from the 5th to the 9th day, but was not studied further. These average tendencies correspond with those observed by Hammett (5) but are not

always exhibited in individual cases. Table III compares the present results with those of Hammett and other workers. In Table IV are shown the individual variations in the change of composition from the 5th to the 9th day. In a small number of instances the protein and ash increased from the 5th to the 9th day, and even more frequently the lactose and fat showed decreases during that period. All components of the milk were quite variable, but the fat fluctuated the most, both in the same individ-

TABLE I.  
*Average Composition of Human Milk at Different Periods.*

Time.	No. of cases.	Protein.			Sugar.			Fat.		
		Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
5 days.	88	1.45	2.83	2.00	4.62	7.37	6.42	0.9	8.2	3.2
9 "	88	1.12	2.65	1.73	4.76	7.65	6.73	1.6	7.1	3.7
3-4 wks.	35	1.03	1.79	1.37	6.17	7.89	7.11	1.4	6.1	3.6
5-6 "	32	0.98	1.57	1.30	5.97	8.33	7.11	1.3	7.6	4.0
7-8 "	14	1.04	1.40	1.21	6.25	7.83	7.11	1.1	7.0	4.0

TABLE II.  
*Composition of Milk of One Woman at Different Periods.*

Days post partum.	Volume.	Sp.gr.	Ash.	Protein.	Sugar.	Fat.
	cc.		per cent	per cent	per cent	per cent
5	60	1.037	0.28	2.20	6.51	0.9
9	113	1.032	0.27	2.00	6.86	5.6
28	33	1.032		1.48	7.05	3.3
42	32	1.032		1.32	6.95	4.4

ual at different periods and in different individuals at corresponding periods. Hammett's individual cases, however, also fail to accord at all times with the group averages.

The present investigation agrees with Hammett's findings that the range of variation of the protein and lactose narrows in later periods, but the fat remains erratic (Table II). The volume of milk obtained by completely emptying one breast was also

extremely variable. On the other hand, the results here reported do not bear out Hammett's observation of a uniform production plane for the individual. Of the 88 cases studied, 58, or 66 per cent, had a protein level consistently above or below the group averages, but only 46, or 52 per cent, maintained a uniform lactose level, and only 36, or 41 per cent, a uniform fat level.

In comparing his results with those of European (1, 2, 4, 12)

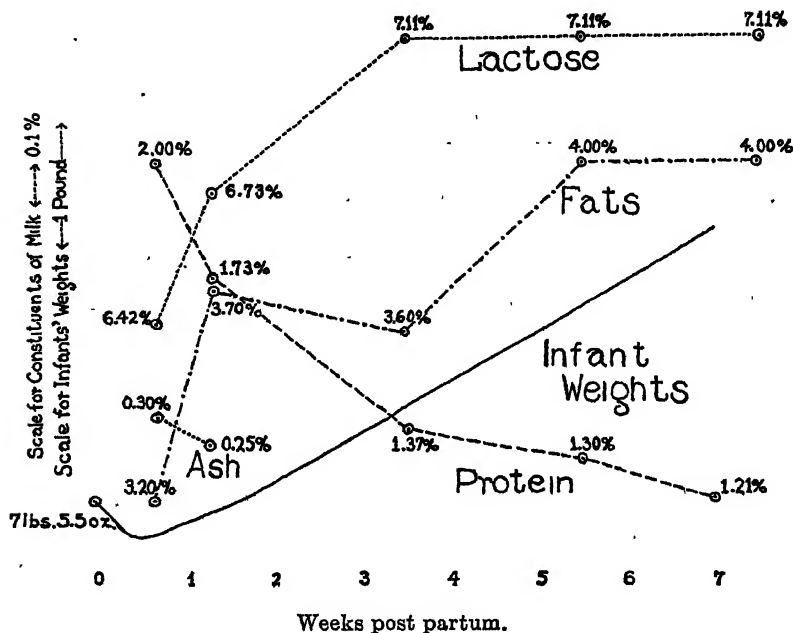


CHART I. Average composition of human milk at different periods, compared with the average weight curve of the infants. The different constituents are plotted on different base lines but the scale is the same in all cases.

and other American (9) investigators, Hammett concludes that the milk of American mothers is higher in lactose and lower in protein than that of European women. The European figures for lactose vary from 3.86 per cent to 5.99 per cent in milk of the 5th to the 11th days post partum, while the American figures quoted are 6.17 and 6.53 per cent. The average for the 5th and 9th days of the 88 cases here discussed is 6.57, agreeing with the other American reports. However, the present results for protein during the

TABLE III.

*Comparison of Averages of Milk Analyses at Different Times after Parturition, Reported by Various Authorities.*

Investigator.	Days post partum.	Ash.	Protein.	Sugar.	Fat.	No. of cases.	Remarks.
		per cent	per cent	per cent	per cent		
Gardner and Fox (3)...	1-6	0.271	2.39	7.03	3.08	179	Average of compilation of results of many other investigators.
Hammett (5).....	3		3.52	5.43	4.34	8	
Holt, Courtney, and Fales (6).....	3-5	0.33	1.96	6.50	3.30	6	
Hammett.....	5		1.74	6.08	2.88	8	Analysis of composite sample.
Present.....	5	0.30	2.00	6.42	3.20	88	Same cases as on 3rd day.
von Gorup-Besanez (4).							
Clemm.....	5-11			3.86			
Tidy.....	5-11			5.14			
Camerer and Söldner (1, 2), Söldner (12)...	5-11		1.77	5.99		13	Composite samples.
Meigs and Marsh (9)...	5-11		1.55	6.53		5	" "
Hammett.....	7		1.61	6.21	3.80	8	Same cases as on 3rd day.
Gardner and Fox.....	7-12	0.237	1.89	6.79	3.42	100	Average of compiled figures.
Holt, Courtney, and Fales.....	5-12	0.31	2.19		3.05	4	Composite sample.
Hammett.....	9		1.69	6.32	3.70	8	Same cases as on 3rd day.
Present.....	9	0.25	1.73	6.73	3.70	88	Same cases as on 5th day.
Schlossmann (11).....	9-10		1.85	6.92	4.23	6	
Hammett.....	11		1.46	6.42	3.38	8	Same cases as on 3rd day.
Schlossmann.....	11-20		1.84	6.89	4.63	25	
Holt, Courtney, and Fales.....	12-30	0.241	1.56		4.37	6	Average of 6 analyses, individual and composite.

TABLE III—*Concluded.*

Investigator.	Days post partum.	Ash.	Protein.	Sugar.	Fat.	No. of cases.	Remarks.
		per cent	per cent	per cent	per cent		
Gardner and Fox.....	13-30	0.216	1.73	6.96	3.94	220	Average of compiled figures.
Present.....	18-31		1.37	7.11	3.60	35	
Schlossmann.....	21-30		1.97	6.77	4.53	41	
“.....	31-40		1.78	6.80	5.41	21	
Present.....	32-45		1.30	7.11	4.00	32	
Schlossmann.....	42-49		1.78	6.80	5.41	13	
Present.....	46-61		1.21	7.11	4.00	14	
Schlossmann.....	51-60		1.59	7.28	4.62	24	

same period average 1.86 per cent, agreeing better with the 1.77 per cent of the European workers than with the 1.55 and 1.62 per cent of the American figures.

When the average weights of the infants were studied it was found that a minimum, averaging 8.4 ounces below birth weight, was reached on the 3rd day. Thereafter a steady increase in weight occurred until an average of 0.4 ounce below birth weight was reached on the 11th day, at which time most cases left the hospital. Of 130 cases studied, 56, or 43 per cent, had regained or passed their birth weight on leaving the hospital, about the 11th day post partum, and 74, or 57 per cent, were still below. The average gain in weight after leaving the hospital was about 9 ounces a week. A careful analysis of the data failed to show any relationship between the composition of the early milk and the gain or loss in weight of the infant.

The different diets fed to the mothers in this investigation had very little observed effect on the composition of the milk (Table V). All four diets were theoretically adequate, the differences being in the addition of an excess of carbohydrate in Groups II and III and an excess of fat in Group IV. The variations in composition of the milk of individuals on any one diet were so much greater than the differences in the respective group averages that it is difficult to draw conclusions.

The average volume of milk was greatest in the high carbohydrate and least in the high fat group. The milk of the group fed on ordinary hospital diet was higher in protein on both the 5th and the 9th days than that of any of the other groups. This diet contained the same quantity but a higher percentage of protein than the others. The milk sugar was higher in both the high carbohydrate and high fat groups on the 5th day than in the control group, but the sugar was about the same on the 9th day in all groups. The high fat diet apparently increased the fat content of the milk on the 5th day but had no effect on the 9th. It would seem, therefore, that feeding of high carbohydrate or fat may raise the milk sugar, or of fat may raise the milk fat, to a slightly higher

TABLE IV.

*Changes in Composition of Milk of Individual Women from 5th to 9th Day Post Partum.*

Group No.	Protein.		Lactose.		Ash.			Fat.		
	No. of cases.		No. of cases.		No. of cases.			No. of cases.		
	Decreased.	Increased.	Decreased.	Increased.	Decreased.	Same.	Increased.	Decreased.	Same.	Increased.
I (control).....	15	2	2	15	16	0	0	6	1	10
II (rice).....	19	1	4	16	17	1	0	6	0	14
III (tapioca).....	25	5	8	23	15	1	2	7	1	23
IV (cream).....	17	2	6	14	3	0	0	11	0	9
All.....	76	10	20	68	51	2	2	30	2	56

level during the period when they tend to be low, but as they naturally increase will not raise them above their normal maxima.

No correlation between the age or parity of the mother and the composition of the milk could be found.

#### SUMMARY.

1. Reports of analyses of breast milk of 88 normal women are presented.

2. The average figures for ash and protein decreased and for lactose and fat increased with the time interval post partum. Individual cases frequently failed to follow this trend.

TABLE V.  
Composition of Early Milk of Women on Different Diets.

Group No.	Days post partum.	No. of cases.	Volume.			Sp.gr.			Ash.			Protein.			Sugar.			Fat.		
			Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.	Minimum.	Maximum.	Average.			
I. (control).	5 17 9	17	cc.	86	59	1.0311	1.0371	1.034	0.28	0.34	0.30	1.752	7.42	1.13	4.627	18.6	1.10	0.9	4.2	3.1
			cc.	14	72	1.028	1.037	1.033	0.20	0.33	0.25	1.592	6.51	1.82	5.377	5.5	6.70	1.7	6.4	3.8
			Average.....				65.5			1.0335			0.275			1.975			6.40	
II. (rice).	5 20 9	20	cc.	13	190	1.027	1.036	1.034	0.24	0.36	0.29	1.502	5.41	1.99	4.757	0.7	6.28	1.6	7.5	3.25
			cc.	11	149	1.028	1.036	1.033	0.21	0.27	0.24	1.332	0.81	1.69	5.867	5.3	6.85	1.9	7.1	3.8
			Average.....				70			1.0335			0.265			1.84			6.565	
III. (tapioca).	5 31 9	31	cc.	6	348	1.030	1.037	1.033	0.25	0.36	0.30	1.602	2.83	1.99	5.297	3.7	6.58	0.9	5.0	3.0
			cc.	10	386	1.029	1.036	1.033	0.18	0.35	0.26	1.122	1.71	1.72	4.767	4.8	6.64	1.9	6.1	3.6
			Average.....				69.5			1.033			0.28			1.865			6.61	
IV. (cream).	5 20 9	20	cc.	13	119	1.027	1.037	1.033				1.452	2.28	1.94	5.797	3.3	6.59	1.4	8.2	3.6
			cc.	6	112	1.027	1.037	1.033				1.422	2.21	1.73	5.407	6.5	6.77	1.6	6.2	3.8
			Average.....				57			1.033						1.835			6.68	

3. A comparison with the results of other investigators has been made.

4. Supplementary feeding of carbohydrate and of fat made only slight differences in the composition of the early milk. The average figures for milk sugar were a little higher in both of the above groups than in the controls and the milk fat was highest in the group receiving supplementary feeding of fat. The average volumes secreted, however, were greater after supplementary feedings of carbohydrate and less after fat feeding than in the controls.

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## THE AVAILABILITY OF ERGOTHIONEINE IN SUPPLEMENTING RATIONS DEFICIENT IN HISTIDINE.

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### INTRODUCTION.

Cox and Rose (1926, b) have fed imidazole derivatives to young rats restricted to diets deficient in histidine. Their purpose was to test whether any preformed imidazole derivative could serve in place of the essential amino acid histidine in the ration. Of the series studied by them, only one,  $\beta$ -4-imidazole lactic acid, enabled the rats to grow. They also attempted to substitute purines, creatinine, and creatine for histidine but found no replacement value in these substances (Cox and Rose, 1926, a). Harrow and Sherwin (1926) have confirmed the findings of Cox and Rose in regard to imidazole lactic acid and have also found that 4-imidazole pyruvic acid can serve in place of histidine, though not as efficiently as the lactic acid derivative. They also claim some replacement by  $\beta$ -4-imidazole acrylic acid (urocanic acid) as opposed to the entirely negative findings of Cox and Rose.

Of the compounds used in the above studies, urocanic acid is the only one associated with animal metabolism that has a structure closely related to histidine. Other known imidazole derivatives identified in animal tissues might possibly support growth if added to a diet deficient in histidine. Growth may result either by sparing dietary histidine that is required for the synthesis of the imidazole derivative in question or by conversion to histidine. Ergothioneine,  $C_9H_{15}N_3O_2S$  (Tanret, 1909), the betaine of 2-thiolhistidine (Barger and Ewins, 1911), recently discovered as a normal component of animal blood (Hunter and Eagles, 1925,

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1927; Benedict, 1925; Benedict, Newton, and Behre, 1926; Eagles and Johnson, 1927; Newton, Benedict, and Dakin, 1927) is such a substance. We have carried out a study of its histidine value in the diet.

#### EXPERIMENTAL.

A healthy litter of ten young albino rats was fed a ration similar in composition to the histidine-deficient diet of Cox and Rose. The histidine-deficient amino acid mixture of Diet D (see Table I) was the same as that previously employed, excepting that silver

TABLE I.  
*Diet D (Histidine-Deficient Diet).*

	gm.
Completely hydrolyzed casein minus histidine.....	14.5
Cystine.....	0.3
Tryptophane.....	0.2
Dextrin.....	40.0
Sucrose.....	15.0
Lard.....	19.0
Cod liver oil.....	5.0
Salt mixture (Osborne and Mendel).*	4.0
Agar.....	2.0
Total.....	100.0

Diet E was prepared by adding 0.545 gm. of ergothioneine to 100 gm. of Diet D. Diet H was prepared by adding 0.5 gm. of histidine monohydrochloride to 100 gm. of Diet D.

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

oxide (Vickery and Leavenworth, 1927) was used to precipitate the histidine. Arginine was not precipitated. A preparation containing sufficient histidine to give a maintenance ration or only slight growth was selected. On such a ration a rat should show an increased growth rate if any added substance has even the slightest value in replacing histidine. The basis of this selection from several preparations was an approximately quantitative determination of the histidine content of the amino acid mixture by means of the intensity of color developed in Hunter's (1922) modification of Knoop's test. Vitamin B was supplied by 200 mg. of dried brewers' yeast daily, instead of the Harris Vitamine

Powder previously used by Cox and Rose. The yeast certainly contributed some histidine to the diet and consequently more rapid growth was obtained on the deficient diets than was expected.

The ergothioneine used was from the preparations of Hunter and Eagles (1925, 1927). The authors gratefully acknowledge their indebtedness to Professor George Hunter of the University of Toronto for the supply of ergothioneine.

The method of caring for the rats was the same as that of Cox and Rose excepting that no preliminary ration was employed.

TABLE II.  
*Food Consumption and Body Weight Changes.*

Days .....	1-4			5-12			13-32			33-52			53-72		
Average daily:	Growth.	Food.	Diet.	Growth.	Food.	Diet.	Growth.	Food.	Diet.	Growth.	Food.	Diet.	Growth.	Food.	Diet.
	gm.	gm.		gm.	gm.		gm.	gm.		gm.	gm.		gm.	gm.	
Rat 949 ♀	-1.5	4.5	H	+0.4	4.5	H	+2.3	5.9	H	+1.5	8.1	H			
" 950 ♂	-2.0	5.1	"	+0.8	5.8	"	+1.4	4.5	"	+0.9	6.2	D			
" 951 ♀	-0.8	5.1	"	+0.5	6.0	"	+2.2	5.6	"	+0.3	5.7	E	+1.9*	7.7	H
" 952 ♀	-1.8	4.8	"	+0.8	6.6	"	+1.5	5.0	"	+0.8	6.4	D			
" 953 ♀	-2.5	4.0	D	-0.6	3.8	D	+0.7	4.0	D	+0.8	5.0	E	+0.5†	3.7	D
" 954 ♀	-2.8	3.5	"	-0.5	5.3	"	+0.6	4.2	"	+2.1	7.0	H	-0.4	5.8	"
" 955 ♂	-2.3	4.5	H	+0.1	4.8	H	+1.5	4.6	H	+0.6	5.8	E	+0.6	5.5	"
" 956 ♂	-1.3	6.0	"	+0.5	5.5	"	+1.1	4.8	"	+1.5	6.5	H			
" 957 ♂	-1.5	6.0	"	+0.6	5.0	"	+2.0	5.4	"	+1.2	6.6	"			
" 958 ♀	-1.5	5.5	"	+0.6	5.5	"	+1.8	5.8	"	+0.3	6.8	D	+2.0‡	7.4	H

\* Only 53 to 68 days.

† An additional period, 73 to 84 days, showed an average daily gain of +2.7 gm. and an average food consumption of 5.3 gm. on Diet H.

‡ Only 53 to 68 days.

Eight of the rats were placed on the deficient diet plus 0.5 per cent of histidine monohydrochloride (Diet H); two, Rats 953 and 954, were started on diets with no added histidine (Diet D). After a period of adjustment, satisfactory growth began in all cases. The two rats on the deficient diet lost weight for a longer time than those receiving histidine and then began to grow at a much slower rate. After 32 days the 0.5 per cent histidine monohydrochloride was removed from the ration of some of the rats, and in the cases of

two of them an equivalent amount (0.545 per cent) of ergothioneine was added. Ergothioneine and histidine were included at this time in the diets of Rats 953 and 954 respectively. The

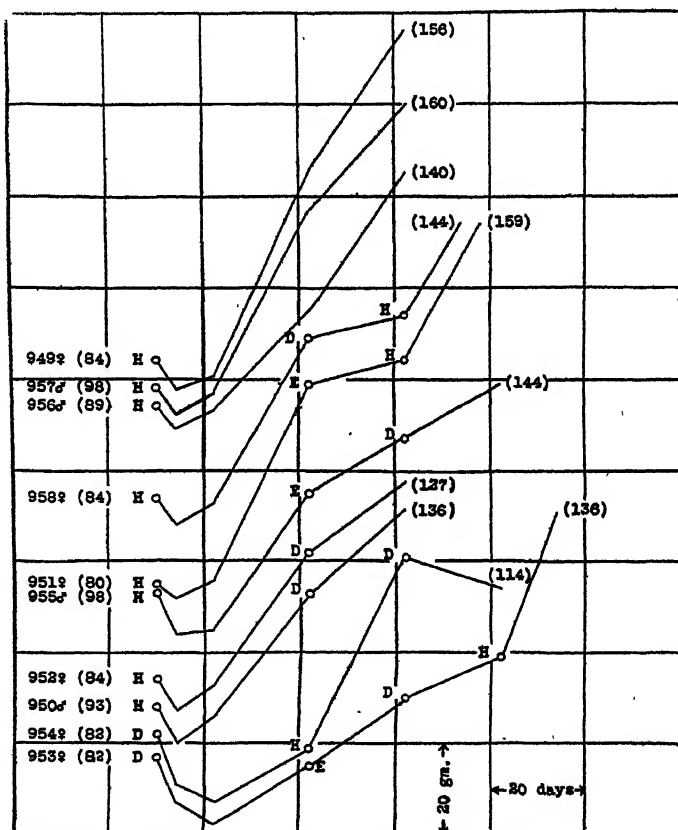


CHART I. The numbers in parentheses at the beginning and end of each growth curve of Chart I are the initial and final weights in gm., respectively, of each rat. Change of ration is indicated on each curve by a small circle. The letters D, E, and H placed near the small circles show that Diets D, E, or H, respectively, were employed in the succeeding period.

experimental period during which the ergothioneine was fed was 20 days. At the end of that time the rats were continued on either the adequate or the deficient diets.

The data of growth and food consumption are presented in Table II, and the growth is shown graphically on Chart I.

#### DISCUSSION.

The data on the average daily consumption of food, which was in all cases offered *ad libitum*, show in general a greater intake of the diet supplemented with histidine. Complete adjustment of all the rats to the rations employed occurred about the 12th day of the experiments. Thereafter the following facts are apparent from Table II.

Growth was always more rapid on the ration supplemented with histidine. When that amino acid was included in the diet the *minimum* growth rate obtained was greater than the *maximum* rate of any rat on either the deficient diet or the ration supplemented with ergothioneine (see Rat 956, period 13 to 32 days, and Rat 950, period 33 to 52 days).

When the rations of the rats were changed, significant alterations in the growth of the animals occurred. Changes of diet from the deficient, or the deficient plus ergothioneine, ration to that supplemented with histidine resulted in the greatest growth rates (Rats 951, 953, 954, and 958). The reverse change from an adequate to the deficient or ergothioneine ration always resulted in retarded growth (Rats 950, 952, and 958; Rats 951 and 955). Changes of diet from the deficient to the deficient plus ergothioneine ration or *vice versa* caused no significant change in the growth rate (Rats 953 and 954).

#### CONCLUSIONS.

No evidence has been found to indicate that ergothioneine can be converted to histidine by the young rat or can diminish the histidine requirement of the growing animal.

The authors acknowledge the suggestion of Dr. R. W. Jackson of Yale University that they undertake this investigation jointly.

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## TOTAL SUGAR OF BLOOD AND URINE.

### II. THE HYDROLYZABLE SUGAR OF BLOOD.

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In a recent paper (1) a method for the estimation of total sugar of blood and urine was described, and values for the hydrolyzable sugar of deproteinized filtrate of whole blood were given. We are now reporting similar values for plasma filtrate.

The separation of corpuscles and plasma of the venous blood of normal adults was made by the technique of Folin and Berglund (2). The non-protein plasma filtrates were prepared by the method of Folin and Wu (3). Clotting occasionally occurred in the plasma of blood drawn from the external jugular vein of animals. In such cases plasma serum was used for the preparation of the protein-free filtrate. In the future it is planned to take mammalian blood from the veins of the extremities, and to use 5 cc. total sugar tubes for the hydrolysis, and 0.5 cc. Ostwald-Folin pipettes for the addition of the acid and alkali, to reduce the volume of blood required.

The free and hydrolyzable sugar were determined by the colorimetric methods of Folin and Wu (4), Benedict (5, 6), Folin (7), an adaptation of Kingsbury's method (8), and a modification of Sumner's method (1). The Folin-Wu values were corrected for proportionality in two ways. The corrections of Oser and Karr (9) were used for all values exceeding 50 mg. per cent, and the corrections described on p. 260 for values from 4.7 to 25 mg. per cent.

Further evidence of the correctness of the original hydrolytic procedure (1) has been obtained. Increasing the concentration of the sulfuric acid solution to 9.0 N led to decreased values for the hydrolyzable sugar. Lengthening the period of hydrolysis from 1½ to 5 hours proved to be of no practical advantage in the analysis



of normal blood. That hydrolysis does not increase the amount of reducing nitrogenous substances in blood filtrate has been demonstrated by means of the ammoniacal copper reagent of Folin and Svedberg (10), which is reduced by creatinine, uric acid, *etc.*, but not by glucose. Several blood filtrates, after hydrolysis by the total sugar method, gave slightly lower reductions than the original filtrates.

Benedict (6) has suggested that substances dissolved from filter paper increase the apparent amount of sugar. Whatman paper used in our experiments was macerated for 2 hours with amounts of distilled water equivalent to the volume of blood filtrate prepared, and the extract carefully analyzed. The amounts of free and hydrolyzable sugar extracted were so small as to be entirely within the limits of experimental error. Similar analyses of extracts of the oxalate cloth in routine use in this laboratory were made after shaking the cloth with the appropriate amount of 0.01 per cent glucose standard for 3 minutes. The effect of the cloth was found to be negligible.

In addition to silicates, ferric iron has an inhibitory action on copper reduction, but the iron must attain a concentration of 0.005 per cent in the glucose standard before it has a measurable effect. Hence, neither potassium hydroxide nor blood filtrates contain sufficient ferric iron to affect the analytical values.

We have assumed, heretofore, that mixtures of sugars are merely additive in their reducing action. This assumption has been verified by analyzing mixtures of sugars having widely divergent equivalents, such as maltose and glucose, and maltose and xylose.

The experiments recorded in Table I show that the major portion of the hydrolyzable sugar of blood is derived from the formed elements, but the plasma also contains a small amount. After a night's fast (Samples 31, 37, 38), the hydrolyzable sugar was still present in both corpuscles and plasma. Folin and Berglund (2) reported negative values for the hydrolyzable sugar of plasma, as determined by the Folin-Wu method. It has been previously pointed out that their results were partly due to the salt effect of the sodium chloride, and partly to the presence of silicates (1).

When blood contains as much free sugar as Sample 25, the possibilities of error in the hydrolyzable sugar estimation are greatly increased, explaining the negative values for this sample

*Distribution Experiments.*

Sample No.	Normal human.	Mg. per cent of sugar.							
		Free.				Hydrolyzable.			
		Folin-Wu.	Benedict.	Folin.	Sunner.	Folin-Wu.	Benedict.	Folin.	Sunner.
7	Whole blood.	98	91			12.5	17		
	Plasma.	101	94.5			3.5	-1		
8	Whole blood.	98		83		12.5		13	
	Plasma.	99.5		115		2		-30.5	
9	Whole blood.	82.5	77	71		15	9	10	
	Plasma.	90	78	78		1.5	1.5	-5	
10	Whole blood.	98	91	83		16	17	13	
	Plasma.	104	96	100		5	12	-15	
13	Whole blood.	73.5	70.5			14	9		
	Plasma.	78	73.5			5	2		
14	Whole blood.	94	84		100.5	11	7		13
	Plasma.	104	90		108	3	2		2.5
	Fasting human.								
31	Whole blood.	101	83	82		8.5	13	0.5*	
	Plasma.	126	106	104		0.5	3	-7*	
37	Whole blood.	98				7			
	Plasma.	90				4			
38	Whole blood.	101		87	116	13		7	7
	Plasma.	108		92	106	2		-1.5	11.5
	Sheep.								
18	Whole blood.		89	85	101		6.5	-1	18
	Plasma serum.		96.5	85	96		1	-1.5	13
20	Whole blood.	86.5	79			7.5	4.5		
	Plasma serum.	119.5	116			4.5	0		
	Rabbit.								
25	Whole blood.	236	235	233		9.5	-4	-13*	
	Plasma serum.	283	269			11.5	-2		
	Dog.								
41	Whole blood.	107		97		6		-1	
	Plasma serum.	131		120		6		0.5	

\* These low hydrolyzable sugar values were later shown to be partly due to silicate effect. The Folin reagents are so sensitive to silicates that the occurrence of negative hydrolyzable sugar values for normal whole blood may be regarded as an almost certain indication of the unsuitability of the potassium hydroxide solution, even though the paraffin lining may apparently be intact.

by the Benedict (5) and Folin methods. On the other hand, we have noticed similar effects at other times with both blood and urine which suggest the possible existence of a part of the free sugar, during hyperglycemia or glycosuria, in a form readily destroyed by the hydrolytic procedure.

The application of Folin's method to the analysis of plasma hydrolysate frequently gave negative results for hydrolyzable sugar, although similar values for whole blood were apparently satisfactory. The sensitive Folin reagent is evidently affected by substances appearing in the plasma filtrate during hydrolysis, resulting in the production of less color from the same amount of sugar.

TABLE II.  
*Effect of Added Glucose on Hydrolyzable Sugar Values.*

Sample No.	Method.	Mg. per cent of sugar.			
		Free.		Hydrolyzable.	
		Blood.	Blood + 100 mg. per cent glucose.	Blood.	Blood + 100 mg. per cent glucose.
45	Folin-Wu.	104.5	203.5	9.5	12.5
	Folin.	90.5	193	9.5	8.5
	Sumner.	103.5	207	24.5	24
47	Folin-Wu.	84	188	7	13
	Folin.	77	180	3.5	-3
	Sumner.	82	184	16.5	13.5

The large amounts of free and hydrolyzable sugar obtained by the Sumner method remained unexplained. They were not due to the effects of creatinine or uric acid. Neither could a separation of a non-glucose reducing substance be secured by treating filtrates with prepared bone-black.

Although we made extensive use of the newer Benedict reagent (6), no results are reported, because other experiments in this laboratory have demonstrated the impossibility of securing accurate results for hydrolyzable sugar by this method. In the near future the data supporting this criticism will be published. The effect of potassium sulfate upon this reagent, and upon Kingsbury's reagent (8) was negligible. Our interest in the latter reagent awaits the development of a suitable modification for blood analysis.

Hydrolyzable sugar values for blood might be due to an increased reduction of the analytical reagents by free sugar, under the influence of catalyzing substances liberated from blood filtrate during the hydrolysis. If this were true, the addition of glucose to blood filtrate might increase the hydrolyzable sugar values. Actually, the deviations of hydrolyzable sugar under these circumstances are no greater than can be accounted for by the necessary experimental error involved in the four separate determinations (Table II).

When blood filtrate was diluted with an equal volume of distilled water (Table III), the deviations of the hydrolyzable sugar,

TABLE III.  
*Effect on Sugar Values of Dilution of Blood Filtrate with Equal Volume of Water.*

Sample No.	Method.	Mg. per cent of sugar.					
		Free.		Hydrolyzable.		Deviation (on basis of original blood).	
		Original.	Diluted filtrate.	Original.	Diluted filtrate.	Free.	Hydrolyzable.
44	Folin-Wu.	99	52.5	8.5	7	6	5.5
	Folin.	88	47	6.5	2	6	-2.5
	Sumner.	102	50.5	12	16.5	-1	21
47	Folin-Wu.	84	48.5	7	4	13	1
	Folin.	77	39.5	3.5	3	2	2.5
	Sumner.	82	48	16.5	16	14	15.5

as determined by the copper methods, again fell within the limits of analytical accuracy; but the Sumner method indicated great increases as a result of the dilution. Hence, the latter method probably gives the least reliable hydrolyzable sugar values.

Our previous attempts to ascertain the effect of alkali hydrolysis upon the hydrolyzable sugar were unsatisfactory because of the lack of suitable methods for analyzing dilute sugar solutions. Reliable results have now been secured by employing the technique of Folin and Svedberg (10). The feebly colored solutions were diluted to a volume of 10 cc., as suggested by Benedict (6).

Studies of the color proportionality for dilute glucose solutions could not be found in the literature. Accordingly, a series of com-

parisons was made against the 25 mg. standard (2 cc. of 0.0025 per cent glucose solution). Averages of the results are given in Table IV. We found a closer approach to theoretical values for the Folin method than for the Folin-Wu method. The undoubted cause of the lack of proportionality in the latter method is the occurrence of a greater blank. If one assumes the color of the blank to be present in both standard and unknown, corrections would be made thus:

Corrected value = actual value  $\times \frac{25 + \text{blank}}{25}$  - blank (in terms of mg. per cent).

TABLE IV.  
*Proportionality of Dilute Glucose Solutions.*

Method.	Glucose in mg. per cent.			
	Theoretical.	Actual.	Corrected for blank.	Average blank.
Folin-Wu.	25	25	25	4.7
	20	20.7	19.9	
	15	16.2	14.6	
	10	11.8	9.3	
	5	8.1	4.9	
Folin.	25	25	25	1.6
	20	18.9	18.4	
	15	13.7	12.9	
	10	9.2	8.2	
	5	5.1	3.8	

By means of this correction the Folin-Wu values become almost exactly proportional. Similar corrections for Folin's method could not be used to improve proportionality, probably because the so called blank, equivalent to 1.6 mg. per cent of glucose, does not represent actual color produced by reduction of the reagent, but other optical effects. For this reason the blank was never used in our calculations. Sumner's method could not be used in the investigation of very dilute sugar solutions because of the lack of sensitivity of the reagent and the pronounced color of the blank.

No previous mention has been made of an obvious source of

error in the copper methods. The glass cups for the Duboscq colorimeters, frequently used in biological analyses, are surrounded by nickeled cylinders of brass, which come in contact with the analytical solution during pouring or rinsing. The use of these cups is inadvisable because the complex molybdic and tungstic

TABLE V.

*Effect of Heating Blood Filtrate, Glycogen, and Glucose Solutions with Alkali.*

Sample No.	Method.	Mg. per cent of sugar.			
		Free.		Hydrolyzable.	
Blood.		Original.	After alkali treatment of blood filtrate.	Original.	After alkali treatment of blood filtrate.
43	Folin-Wu.	102	5.5	11.5	7
	Folin.	90.5	3	11.5	6
45	Folin-Wu.	104.5	7.5	9.5	9
	Folin.	90.5	5	9.5	7
47	Folin-Wu.	84	6	7	7
	Folin.	77	7.5	3.5	7.5
Glycogen.	Method.	Free after acid hydrolysis.		Free after acid hydrolysis of glycogen previously treated with alkali.	
			Equivalent to:		Equivalent to:
		mg. per cc.	mg. per cent	mg. per cc.	mg. per cent
1	Folin-Wu.	0.0195	19.5	0.0105	10.5
	Folin.	0.016	16	0.0065	6.5
2	Folin-Wu.	0.027	27	0.0150	15
	Folin.	0.0245	24.5	0.0140	14
Glucose.	Method.	Free.		Free after alkali treatment.	
			Equivalent to:		Equivalent to:
		mg. per cc.	mg. per cent	mg. per cc.	mg. per cent
1	Folin-Wu.	0.100	100	0.0025	2.5
	Folin.	0.100	100	0.0025	2.5
2	Folin-Wu.	0.100	100	0.0045	4.5
	Folin.	0.100	100	0.0025	2.5

acid reagents are readily reduced by metallic nickel and copper, just as they are by cuprous oxide. Careful observation of the interior of the cup, containing such acid reagents, will show a drainage of deeply colored liquid from the metal rim. It is much better to use all-glass cups for sugar analyses, especially when dealing with fermented filtrates, *etc.*

As to the possible nature of the hydrolyzable sugar of blood filtrate, it will be recalled that glycogen, if present in blood, will at least partly escape precipitation with the proteins in the preparation of the filtrate (1). Moreover, the hydrolyzable sugar is largely contained within the formed elements of the blood, as would be expected if the material were glycogen. The results recorded in Table V show the effect of heating 8 cc. portions of blood filtrate, of 0.01 per cent glucose solution, and of 0.005 per cent glycogen<sup>1</sup> solution with 1 cc. of 2.6 N potassium hydroxide solution for 1½ hours on the water bath. (It was impractical to use the greater concentrations of alkali which are claimed to have no effect on glycogen (11).) After cooling, the mixtures were neutralized with 1 cc. of 2.6 N sulfuric acid. In the case of the glycogen solution it was, of course, necessary to submit the original and alkali-treated solutions to hydrolysis, by the total sugar method, before the sugar was determined. The slight salt effect, due to the increased amounts of potassium sulfate in the hydrolyzed, alkali-treated, analytical samples, was compensated for by using similar amounts of acid and alkali in the standard glucose solutions.

The small amount of color generated by glucose solutions after alkali treatment was very little more than the absolute blank in the Folin method. Therefore, one is justified in claiming the destruction of at least 99 per cent of the glucose. The glycogen solutions, while more resistant to alkali treatment, were markedly affected by the relatively dilute alkali employed. A little less than 50 per cent of the glycogen was destroyed, or changed in such fashion as to prevent the formation of reducing glucose by acid hydrolysis.

The alkali treatment of blood filtrate shows distinctly that the free sugar of blood is not entirely like glucose, since 5 to 7 per cent of the free sugar by the Folin-Wu method, and 3 to 9.5 per cent by the Folin method, remain after the alkali treatment. These values approximate those secured for residual reduction of blood filtrate after fermentation. The hydrolyzable sugar of blood filtrate is usually partly destroyed, as in the case of glycogen, but the losses are quite variable because of the necessarily large experimental error involved. Grossly, the material may be said to resemble glycogen, being at least as resistant to the action of alkali.

<sup>1</sup> C. P. Eastman Kodak Company.

An attempt was made to ascertain whether the sugar, liberated from blood filtrate during the total sugar procedure, resembled glucose in being fermented by yeast. After careful consideration of short period methods of "fermentation" (10, 12), we decided to employ the method of Folin and Svedberg (10). Our work was well under way when Benedict's proposed modification appeared (6). His process is intended chiefly for use with whole blood rather than blood filtrate. Hence it was decided to continue with the Folin-Svedberg method. Washing the yeast, as Somogyi and Benedict advise, is undoubtedly better and would give lower blanks, but the nature of our reported results would hardly be

TABLE VI.  
*Effect of Fermentation of Hydrollysate on Hydrolyzable Sugar Values.*

Sample No.	Method.	Mg. per cent of sugar.			
		Free.		Hydrolyzable.	
		Original.	After fermentation.	Original.	After fermentation.
21	Folin-Wu.	90	10	7	4
	Folin.	81	7	9.5	7
23	Folin-Wu.	96	7	9	8
	Folin.	86.5	3	8.5	8
24	Folin-Wu.	88	8	10	3.5
	Folin.	74	4	6	4.5
36	Folin-Wu.	93	8.5	7.5	5
	Folin.	78	5.5	-1*	3

\* Silicate effect.

altered. In a very recent paper, Somogyi (13) has recommended a new procedure for filtrate. At a later date we intend to repeat some of our experiments, using this technique.

The blanks for the yeast, to be used in correcting the free sugar values, were determined upon freshly prepared 0.01 per cent glucose standard. Similar blanks for the total sugar determinations were made on the glucose standard plus the requisite amount of potassium sulfate (added as alkali and acid). In the presence of the salt, yeast apparently contributed slightly more reducing material, the average increase being 1.5 mg. per cent of sugar for the series of experiments.



The results of these experiments (Table VI) indicate that a large proportion of the hydrolyzed sugar is not "fermented." It would be more accurate to say that it is neither absorbed nor adsorbed by the yeast. The results are sufficiently variable to cast doubt upon the validity of the fermentation test under these circumstances.

As a check upon the foregoing experiments, glycogen solution was also fermented (Table VII). In Experiments 1 and 2 a 0.005 per cent, freshly prepared, unpreserved solution of glycogen was used; and in Experiment 3 a solution of 10 times this strength. The solutions were first hydrolyzed by the total sugar method and the fermentation carried out as previously described,

TABLE VII.  
*Effect of Fermentation on Glycogen Hydrolysate.*

Sample No.	Method.	Free sugar.			
		Before fermentation.		After fermentation.	
			Equivalent to:		Equivalent to:
		mg. per cc.	mg. per cent	mg. per cc.	mg. per cent
1	Folin-Wu.	0.017	17	0.0075	7.5
	Folin.	0.0175	17.5	0.004	4
2	Folin-Wu.	0.020	20	0.005	5
	Folin.	0.0165	16.5	0.0025	2.5
3	Folin-Wu.	0.192	192	0.057	57
	Folin.	0.159	159	0.069	69

except that the blanks for the yeast were determined on glycogen solutions rather than on glucose solutions. Corrections have been made for the small increased yeast blank due to the salt effect. The results show that a variable portion, but approximately one-third, of the reducing sugar set free by the hydrolysis of glycogen is not removed by the yeast. Therefore the residues of reducing sugar from fermented hydrolysates of blood filtrates do not constitute evidence against the possibility of the hydrolyzable sugar of blood being glycogen. The failure of yeast to remove all of the hydrolyzed glycogen complicates the interpretation of the non-fermentable residues of blood sugar.

A further example of the complex conditions to be encountered in fermented blood filtrates is given by several experiments in

which fermentation preceded hydrolysis (Table VIII). The purpose of these experiments was to determine whether the hydrolyzable sugar could be removed from the filtrate in a similar fashion to glucose. It was not possible to secure a definite answer to this question. After the calculations had been made for Sample 44, it was evident that some hydrolyzable sugar had been extracted from the yeast by the blood filtrate. In the two subsequent experiments, in addition to the customary blanks for the yeast, 0.01 per cent freshly prepared solution of glucose was fermented, and the filtrate of this mixture hydrolyzed by the total sugar method. Large amounts of hydrolyzable sugar were found to be present in these solutions. Even after the large blanks had been

TABLE VIII.  
*Effect of Fermentation of the Filtrate before Hydrolysis.*

Sample No.	Method.	Mg. per cent of sugar.					
		Free.		Hydrolyzable.		Yeast blank.	Increases in blanks from hydrolysis (equivalent to hydrolyzable sugar).
		Original.	After fermentation.	Original.	After fermentation.		
44	Folin-Wu.	99	9.5	8.5	18.5	5.8	
	Folin.	88	4	6.5	16.5	3.5	
46	Folin-Wu.	90	12	10	12	9.8	20.4
	Folin.	83	6	3	11	8.3	18
48	Folin-Wu.	94	18	10	23	13.1	26.7
	Folin.	77	3	6	22	10.5	11.2

subtracted, more than the original amounts of hydrolyzable sugar were present in the fermented blood filtrates. These results confirm the validity of Somogyi's recommendation that the yeast be thoroughly washed.

In Table IX we present further data on the hydrolyzable sugar of human whole blood. We have also included the average values for the hydrolyzable sugar of normal whole blood and plasma.

The leucemic blood (Sample 49), obtained through the courtesy of Dr. Hugh G. Jeter of the University Hospital, had a white cell count of 130,000, and contained more hydrolyzable sugar than normal blood. While approximately 20 times the normal number

TABLE IX.  
*Hydrolyzable Sugar of Human Whole Blood.*

Sample No.	Mg. per cent of sugar.							
	Free.				Hydrolyzable.			
	Folin-Wu.	Benedict.	Folin.	Sumner.	Folin-Wu.	Benedict.	Folin.	Sumner.
2	80				9			
3	98	89		91.5	6	8		10.5
4	88.5	88.5			10	9		
6	87	86			6	5		
7	98	91			12.5	17		
8	98	91	83		12.5	7	13	
9	82.5	77	71		15	9	10	
10	98	91	83		16	17	13	
13	73.5	70.5			14	9		
14	94	84		100.5	11	7		13
19	91			107	7			1
21	90		81		7		9.5	
23	96		86.5		9		8.5	
24	88		74		10		6	
31	101	83	82		8.5	13	0.5*	
33	99				11.5			
34	76		63		9		1*	
35	95		84		6.5		-1*	
36	93		78		7.5		-1*	
37	98		85		7		0*	
38	101		87	116	13		7	7
39	81		70	81	7.5		2	15
40	114.5		103	126	9.5		5	9.5
42	99		88	99	10		9.5	19
43	102		90.5	102.5	11.5		11.5	16.5
44	99		88	102	8.5		6.5	12
45	104.5		90.5	103.5	9.5		9.5	24.5
46	90		83	95	10		3	15
47	84		77	82	7		3.5	16.5
48	94		77	100.5	10		6	11.5
49 (leucemia).	68		54	62	12		15.5	36.5
Average normal values.								
Whole blood.					10	11	8	14
Plasma.					3	3	Indeterminate.	Insufficient data.

\* Values for hydrolyzable sugar made with alkali which had a silica effect. The reported values are approximately 9 mg. per cent too low.

of white cells were present the hydrolyzable sugar was but twice the normal amount by the Folin method, and only slightly increased by the Folin-Wu method. If the analyses of glycogen in mammalian leucocytes by de Haan (14) be taken as a basis for calculation, Sample 49 should have contained approximately 45 mg. per cent more glycogen than normal samples because of the increased white cell count. This amount of glycogen would increase the hydrolyzable sugar by approximately 20 mg. per cent of glucose. Some glycogen was undoubtedly lost in the protein precipitate (1), and pathologic white blood cells might contain less glycogen than normal cells. According to de Haan's findings normal blood should contain approximately 2 to 4 mg. per cent of glycogen, of white cell origin. This amount of glycogen would give, by our total sugar method, a hydrolyzable sugar value of 1 to 2 mg. per cent of glucose. Normal blood contains 5 to 10 times this amount of hydrolyzable sugar, the greater part of which cannot at present be traced to a leucocytic origin.

Since glycogen has never been detected in erythrocytes, we conclude that the greater portion of the hydrolyzable sugar of blood is not glycogen, or else true analytical values for the glycogen of blood cells have not been attained. Perhaps the use of concentrated alkali in the separation of small amounts of glycogen leads to some destruction. We hope by isolation experiments to be able to secure added data on the nature of the hydrolyzable sugar of blood. Recently Fontes and Thivolle (15) and Bigwood and Wuillot (16) have published data concerning the nature of "combined sugar" of blood filtrates, but the different technique employed prevents direct comparison with our results.

#### SUMMARY.

Further investigations concerning the suitability of the total blood sugar method are reported. Evidence is presented to show that the hydrolyzable sugar of blood actually exists, and is not due to analytical errors. Values are given for the hydrolyzable sugar of plasma. Folin's method cannot be used for these plasma determinations. Essentials for the analysis of dilute sugar solutions are discussed. The effects of fermentation and of alkali

hydrolysis upon the hydrolyzable sugar indicate that it resembles glycogen, but the latter has never been found in sufficient quantities in normal blood to account for more than a small part of the hydrolyzable sugar (14, 17-20). A sample of leucemic blood contained more hydrolyzable sugar, but not enough to indicate the white cells as the chief source of this material.

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## CUTANEOUS AND VENOUS BLOOD SUGAR CURVES.

### I. IN NORMAL INDIVIDUALS AFTER INSULIN AND IN LIVER DISEASE.

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In an attempt to define more accurately the nature of the disturbances of carbohydrate metabolism in mild diabetics and persons with benign glycosuria, the authors have investigated the arterial (cutaneous) and venous blood sugar curves of a variety of subjects after the administration of carbohydrate food.

#### HISTORICAL.

The fact that the concentration of glucose in systemic arterial blood exceeds that in the systemic venous blood was first demonstrated by Chauveau (1) in 1856, before Claude Bernard had discovered glycogen or was even willing to admit the presence of glucose in systemic blood. Perhaps for this very reason the difference between arterial and venous blood was overlooked by most subsequent observers, an oversight that has had a definite influence on the history of carbohydrate metabolism studies and has led to many unnecessary controversies.

Bang (2) in his important monograph, published in 1913 and, unfortunately, never translated into English, was aware of the arterial-venous difference and was influenced by its existence. In this work he presented a method for the determination of sugar in 0.2 cc. of blood or less. Almost simultaneously Lewis and Benedict (3), in this country, described a method suitable for the analysis of about 2 cc. of blood. These two methods for the first time made blood sugar determinations practical clinical procedures. Bang's procedure was generally adopted in Europe, and became the standard on which other techniques were modeled. It was suitable for the analysis of such small amounts of blood as could be obtained by puncture of the skin of the finger or the ear. Benedict's method was as generally accepted in America and subsequent methods developed on this side of the Atlantic followed Benedict's lead in requiring 1 or 2 cc. of blood or its equivalent, rendering them applicable only to the study of venous blood.

## 270 Cutaneous and Venous Blood Sugar. I

Hagedorn (4) and Foster (5) have shown that cutaneous blood is identical with arterial blood as far as its sugar content is concerned. It therefore follows that European investigators have in general examined arterial blood, while Americans have examined venous blood in their clinical studies.

Although Bang (2) was aware of the difference between arterial and venous blood and, presumably, was influenced by this knowledge, there is little evidence that subsequent workers chose their methods of study with any consciousness that such a difference existed until Hagedorn (4) published his investigations of the subject in 1920. Folin and Berglund (6), indeed, offered as an explanation of the greater alimentary hyperglycemia found by European workers, the theory that puncture of the finger or ear caused greater pain than venous puncture, and, therefore, greater nervousness. After the publication of Hagedorn's studies Scandinavian investigators, at least, began to appreciate the importance of distinguishing between cutaneous and venous blood. It was not, however, until 1923 that the distinction was recognized in this country by Foster (5), who confirmed in every respect the observations of Chauveau (1) and Hagedorn (4). Since then Holst (7), Lawrence (8), Lundsgaard and Holbøll (9), and Rabinowitch (10) have made studies of the alimentary arterial venous blood sugar difference in normal persons and in diabetics.

All workers agree that in the postabsorptive state the concentrations of sugar in arterial and venous blood are identical. After the ingestion of carbohydrate both rise. The rise of the venous curve is checked, however, before that of the arterial and falls more rapidly. At a variable period after the meal the venous sugar sinks not only to, but actually below, the initial postabsorptive level. This is the postalimentary hypoglycemia, first described by Bang (2). The cutaneous curve reaches the initial level more slowly than does the venous curve, but, like the latter, eventually tends to fall slightly below it. The two curves then converge at the fasting level. Holst (7), Lawrence (8), Lundsgaard and Holbøll (9), and Rabinowitch (10) have all found that in the severe diabetic the alimentary arterial-venous difference may be absent or greatly diminished. Lundsgaard and Holbøll (9) claim that the difference can be restored to the diabetic by the administration of insulin.

Cori and Cori (11) have, by a clever series of experiments, demonstrated the mechanisms which determine the form of the alimentary blood sugar curve and its variations under different conditions. At all times glucose is being absorbed from the blood by the muscles and other tissues to be stored as glycogen, which is, in turn, continually decomposed to lactic acid and oxidized. The rate of removal is, in the resting, fasting state, however, too small to be readily distinguished. The blood sugar is as continuously replenished from the hepatic stores of glycogen, which are obtained from ingested preformed glucose or by the transformation in the liver of other carbohydrates or protein. The initial rise of arterial and venous blood sugar after the ingestion of glucose is an expression of the absorption of sugar from the alimentary tract. The subsequent reduction of the blood

sugar to the normal level is the result of two factors: (1) the removal of sugar by the liver to form glycogen; (2) the removal of sugar by the tissues, and especially muscle, for the formation of tissue glycogen and for combustion. It is the latter process that is evidenced in the arterial-venous difference. Insulin appears to exert its chief effect in promoting removal and consumption of sugar by the tissues.

Because the blood sugar begins to fall while absorption of carbohydrate is still at its height and because alimentary hyperglycemia is eventually succeeded by a period of hypoglycemia, it may be inferred that the mechanisms for the removal of sugar from the blood and its disposal have a certain inertia. They are activated to their full extent only after the blood sugar has risen measurably above the normal level and are not again checked until it has fallen distinctly below this level.

### *Investigative Procedure.*

For the analysis of blood a micro modification of Benedict's 1925 (12) procedure was employed. Earlier attempts to adapt the method of Folin and Wu (13) to micro analysis were not entirely successful, although it has been so employed by Foster (5) and others. Benedict's method has a definite advantage in the fact that the color developed is more intense. His proposal that benzene be vaporized in the tubes during the reduction procedure proved entirely successful in our hands and obviated the necessity of using special tubes. When the method began to be applied more generally, however, it proved simpler to have tubes constructed which were especially adapted to the micro procedure. These will be described below.

In 1926 Benedict (14) modified his method to meet certain objections. He pointed out that bisulfite, which had been used in the copper solution was not a salt of constant composition and substituted sulfite. He also added ammonium salts to keep the reduced copper in solution. Finally he increased the acidity and the specific gravity of the arsenophosphotungstate color reagent by the addition of HCl and NaCl. An early attempt to adapt this method directly to micro analysis proved unsuccessful because of the frequent appearance of precipitates. No such difficulty was encountered when it was employed for the analysis of the usual volumes of blood filtrate. More recently, with the smaller tubes no precipitation has occurred. The 1925 method had, however, proved so satisfactory that it seemed best to continue it.



On examination of the results of all the studies together, an interesting fact appears. The method has been employed by all four of the authors in different phases of the investigation and by three technicians in the same laboratory. It has been found by all to check perfectly with the similar macro procedure at all times. As it was employed glucose solutions equivalent to filtrates from bloods containing glucose varying from 50 to 400 mg. per 100 cc. can be compared without error in the colorimeter. When the results of the various studies are compared, it is found that the normal values obtained in 1926 are all consistently lower than those of 1927 and 1928. The difference was first ascribed to the fact that the subjects of the 1927 series were given glucose, while those of 1926 were given mixed meals. The fact that fasting values, as well as those after food, were low was overlooked.

The only explanation that can be offered for the discrepancy is that different reagents were used for the first series. Different supplies of bisulfite may have been responsible. When Benedict originally proposed the method he pointed out that it gave values consistently lower and probably more correct than the Folin-Wu procedure. This we verified in the 1926 series. In the later series, however, the values obtained are quite as high as those usually obtained by the Folin-Wu method.

#### *Analytical Procedure.*

In a pipette carefully calibrated to contain 0.2 cc. blood was secured from the ear or finger tip of the subject. Venous blood was withdrawn by means of a small hypodermic syringe, transferred at once to a clean glass container, and thence drawn into the pipette. The blood thus obtained is immediately delivered into 1.6 cc. of  $N/12$   $H_2SO_4$  in a conical centrifuge tube and the pipette carefully washed out by drawing the acid into it two or three times. The blood is well mixed with the acid by means of a small footed stirring rod. When it is completely laked 0.2 cc. of 10 per cent sodium tungstate is added, the whole is thoroughly mixed with the stirring rod, and, after standing a few minutes is centrifuged. Sufficient protein-free supernatant fluid for duplicate blood sugar determinations can be secured from 0.2 cc. of blood treated in this manner.

0.5 cc. of the supernatant fluid is transferred to a Folin-Wu

blood sugar tube, an ordinary test-tube of moderate diameter, calibrated at 6.25, 12.5, and 25.0 cc., or, best of all, one of the special tubes described below. In a similar tube is placed 0.5 cc. of a standard glucose solution containing 0.1 gm. of glucose per liter. Standard and unknown are thereafter treated alike. To each is added 0.5 cc. of the Benedict copper solution. Unless the special tube is used, 4 drops of benzene must then be added and the tubes stoppered with cotton. They are then placed in boiling water, where they are allowed to stand for 5 minutes. At the end of this time they are cooled in cold water for 2 minutes. After this 0.5 cc. of the arsenophosphotungstate solution is introduced. The mixture is allowed to stand 10 minutes longer to insure complete solution of the reduced copper. In some instances this must be aided by agitation of the tube.

At the end of this time standard and unknown are diluted and compared in a colorimeter. The colors of standard and unknown should not differ by more than 30 per cent. It is, therefore, necessary either to employ several standards of different strengths or to use a combination of dilution and comparison colorimetry as advocated by Rothberg and Evans (15). If the blood sugar is less than 100 mg. per 100 cc. the color is so faint as to make colorimetric comparison difficult, even if it is diluted only to 12.5 cc. It is, therefore, advisable, if Rothberg and Evans or Folin-Wu tubes are used to mark them at 6.25 cc.

Special tubes have been devised which greatly facilitate the procedure. These are similar to the Rothberg and Evans (15) modification of the Folin-Wu tube, but smaller. They are so made that when 1 cc. of fluid is introduced, the upper level of the liquid lies in the constricted portion of the tube which must not exceed 6 mm. in diameter. The tubes are graduated at 1.25 cc. intervals from 6.25 to 25.0 cc. With these tubes standard and unknown can be diluted till their colors are a convenient depth and almost match before comparison in the colorimeter. By this combination of dilution and comparison colorimetry bloods varying in sugar concentration from 50 to 400 mg. per 100 cc. can be analyzed with a single standard without error. The use of benzene to prevent reoxidation of the reduced copper is also unnecessary.

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### *Calculation of Results.*

$\frac{100 SV, Q}{RV_s}$  = mg. of glucose per 100 cc. of blood, where  $S$  and  $R$  = readings of standard and unknown respectively;  $V_s$  and  $V_r$  = the volume in cc. to which standard and unknown, respectively, were finally diluted;  $Q$  = the strength of the standard solution in gm. of glucose per liter.

### *Treatment of Material.*

In all experiments on alimentary hyperglycemia, blood was secured in the morning in the postabsorptive state, at least 12 hours after the last preceding meal. As soon as possible after the preliminary sugar determination, the subject was given a known dose of glucose dissolved in the diluted juice of an orange or a lemon. After this the blood sugar was again determined at 30 minute intervals for from 90 to 120 minutes. When insulin was given without food the blood sugar was determined before and at 30 minute intervals after insulin injection.

An attempt was made, in every case, to secure both venous and arterial samples as nearly as possible simultaneously. The venous sample was usually taken first. The total time spent in withdrawing the two bloods seldom exceeded 3 minutes.

Urines from all subjects were tested for sugar by Benedict's qualitative test before and after each experiment. None of the normal subjects showed glycosuria.

### *Normal Alimentary Reaction of Arterial and Venous Blood.*

The data obtained from normal individuals are collected together in Table I. In all twenty-four studies were made on twenty normal adults (medical students, interns, and laboratory workers). The ten of the 1927 series were studied for 2 hours after a meal consisting of 50 gm. of glucose dissolved in 200 cc. of water with the juice of an orange or lemon. The five of the 1926 series were studied seven times for 1.5 hours after mixed meals containing 75 to 115 gm. of carbohydrate. In two additional experiments one of the subjects received on one occasion 75 gm., on another 150 gm. of glucose. The five of the 1928 series were studied for 1.5 hours after mixed meals containing from 70 to 100 gm. of carbohydrate.

These various procedures were chosen because the individual

series were intended to serve as controls for different types of experiments. As has been pointed out in the discussion of analytical procedure, in the 1926 series, as a whole, all values lie distinctly below those of other years. This is probably due neither to technical errors nor to peculiarities of individuals examined during the year 1926, but to differences in the chemicals (probably the bisulfite) used in the preparation of the reagents. Separate controls were made at intervals in each series to prove that intensity of color varied directly with sugar concentration. The only possible

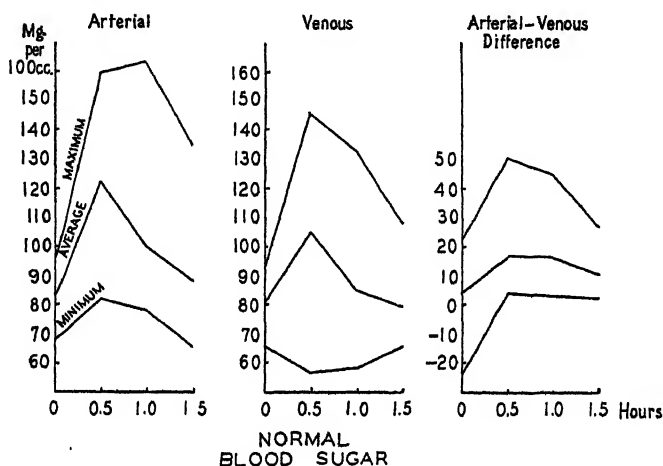


Fig. 1.

conclusion then, is that the 1926 reagents were less susceptible to non-glucose reducing substances than were subsequent reagents. If the 1926 values are all increased by 25 mg. per 100 cc. they agree well with those of other years both in fasting level and in degree and duration of hyperglycemic reaction.

Examination and comparison of all the series after the 1926 values have been increased by 25 mg. show no demonstrable difference in the hyperglycemic reaction after 50 gm. of glucose and that after a mixed breakfast. The variability of the reaction after either type of meal is too great to permit one to make any clear distinction.

TABLE I.  
*Blood Sugar Values of Normal Individuals.*

Mg. per 100 cc.

Subject No.	Fasting.			Time after meal.												Meal.	
	Cutaneous.	Venous.	Difference.	30 min.			60 min.			90 min.			120 min.				
				Cutaneous.	Venous.	Difference.	Cutaneous.	Venous.	Difference.	Cutaneous.	Venous.	Difference.	Cutaneous.	Venous.	Difference.		
1927																	Glucose.
1	78	70	8	121	107	14	74	75	-1	86	73	13	87	70	17	50 gm.	
2	74	71	3	129	115	14	97	83	14	67	66	1	68	66	2	50 "	
3	73	74	-1	108	78	30	96	97	-1	94	70	24	84	73	11	50 "	
4	94	89	5	125	101	24	107	89	18	104	86	18	88	81	7	50 "	
5	85	78	7	120	112	8	106	103	3	102	94	18	81	88	-7	50 "	
6	83	66	2	98	88	10	105	89	16	77	98	-21	85	79	6	50 "	
7	108*	74	34	127	112	15	83	63	20	81	71	10	76	62	14	50 "	
8	95†	89	6	128	109	19	144	128	16	169	132	37	124	125	-1	50 "	
9	93	71	22	158	108	50	102	72	30	84	70	14	77	66	11	50 "	
10	85	83	2	149	128	21	92	78	14	88	72	16	85	77	8	50 "	
Maximum.	103	89	34	158	128	50	144	123	30	169	132	37	124	125	17		
Minimum.	68	66	-1	98	78	8	74	63	-1	67	66	-21	68	62	-7		
Average.	85	77	9	126	106	21	101	88	13	95	83	12	86	79	7		

1 a b c d  2 a b 3 4 5	Calorimetric.												101 “	
	60 56 59 56 51 63 68 56 70	59 64 58 56 50 63 65 56 58	1 -8 1 0 1 0 3 0 12	97 113 103 83 59 105 96 78 102	93 103 83 70 53 73 92 66 90	4 10 20 11 6 32 4 12 12	74 85 91 77 55 61 61 76 95	52 75 46 66 52 49 46 64 75	22 10 45 11 3 12 15 12 20	62 54 58 40 72 41 52 43 98	52 45 33 61 52 49 42 42 78	10 9 25 11 -12 -8 10 1 20		102 gm. 102 “ 75 “ 170 “ 74 “ 74 “ 96 “ 116 “ 101 “
Maximum. Minimum. Average.	70 51 60 85	65 50 59 84	12 -8 1 118	113 59 93 118	103 53 80 105	32 4 12	95 51 75 100	75 46 58 83	45 3 17 83	98 40 58 83	78 42 50 75	25 -12 7		
1948	Mixed meal. Carbohydrate.													
	85 gm.													
	1	75	78	-3	112	89	23	92	81	11	83	74	11	97 “
	2	69	93	-24	104	97	7	90	72	18	96	75	21	73 “
	3	90	84	14	77	57	20	85	59	26	96	108	27	80 “
4	97	90	7	160	146	14	163	132	31	135	98	5	67 “	
5	83	77	6	149	135	14	132	118	14	103	108	27		
Maximum. Minimum. Average.	97 75 83	93 77 84	14 -24 0	160 77 120	146 57 105	23 7 16	163 85 112	132 59 92	31 11 20	135 83 104	108 74 94	27 5 16		

\* Some trouble and delay was encountered in securing the venous blood. This may have caused sufficient nervousness to disturb the blood sugar results.

† This patient felt faint just before the test.

Fig. 1 represents graphically the extent of variation of arterial and venous blood sugar changes in the whole series and the differences between the two. In this and subsequent figures 25 mg. have been added to all the 1926 values. As it is not absolute values but changes resulting from disease or other factors that are under consideration, such correction seems justified. Actual determined values are given in all tables.

In the fasting state the arterial venous difference was less than 10 in nineteen out of twenty-four determinations. In nine instances a difference of more than 5 mg. was observed. In only four of these did it exceed 8 mg. Even at rest in the fasting state the removal of sugar by the tissues can apparently become accelerated to a point where it is easily perceptible. At times it may become quite striking. The value of 34 obtained on Subject 7 of the 1927 series should probably be discounted because some difficulty was experienced in securing the first venous sample. 15 minutes altogether were consumed in taking both venous and arterial blood and the subject experienced considerable pain. No such factors, however, were active in the experiment on Subject 9 of this series or Subject 3 of the 1928 series in which differences of 22 and 14 respectively were observed.

In two instances negative (arterial < venous) differences of significant magnitude (-8 and -24) occurred. Such negative differences will be encountered in other connections with sufficient frequency to make it evident, even if duplicates and controls had not been carefully carried out, that they are not due to technical errors. They have also been reported by others. The obvious explanation for them is that at times sugar is given off by the tissues to the blood, an idea that is contrary to physiological conceptions of carbohydrate metabolism. Bollman, Mann, and Magath (16) have shown that glycogen of muscles is not available for the restoration of the blood sugar in the hypoglycemia following removal of the liver. Cori and Cori (11) have also been unable to demonstrate such a reversal of the normal sugar current.

Folin and associates (17) have suggested that sugar may be temporarily absorbed and held by tissues in the free state, to be liberated into the blood later for distribution. It may be such sugar that is evidencing itself in these experiments and not sugar derived from tissue glycogen. However, there is no apparent reason why tissues in the fasting state should contain an excess

of free sugar. General hemoconcentration could hardly explain such large differences. On the whole they would rather seem to indicate that, under certain conditions, some part of the carbohydrate in tissues can be mobilized into the blood.

In most instances both arterial and venous sugars rise quite rapidly, reaching their maxima in half an hour, and are already on the descent at the end of an hour. The height to which they rise is variable, a variation that is probably partly the result of the procedure employed. Hansen (18), Hatlehol (19), and others who have studied cutaneous blood sugar at frequent intervals have shown that the peak of the alimentary curve is quite sharp and occurs at a variable interval after meals, usually less than an hour. If the peak is detected in any of the curves here presented it is due to a mere coincidence. One can only conclude from these studies that the chances of detecting the maximum rise are greater 30 minutes than they are 60 minutes after a meal. In four instances the values at 60 and 90 minutes were higher than those at 30. The subject of one of these experiments, No. 8, 1927, was very nervous and almost fainted when the first blood sample was taken. His curve is the highest and most prolonged in the series, attaining a maximum of 169 only after 90 minutes and remaining as high as 124 at the end of 2 hours. Although he seemed normal and his nervousness passed off almost immediately, it is doubtful whether this curve should be included in the normal group. The curve for Subject 4 of the 1928 series, another delayed curve, also attained an unusual height. Both of the other delayed curves, Subject 6, 1927, and Subject 3, 1928, showed unusually small fluctuations, permitting little significance to be attached to the delay.

In no experiment did the venous sugar rise as high as the arterial, although significant negative differences were observed in three instances. The maximal, minimal, and average rises of arterial and venous sugars and the differences between them were:

	Maximum. mg. per 100 cc.	Minimum. mg. per 100 cc.	Average. mg. per 100 cc.
Arterial rise.....	74	6	41
Venous rise, actual.....	58	-7	27
“ “ above fasting arterial..	52	-15	23
Arterial-venous difference.....	50	6	22
Increase of difference over fasting...	44	-14	18



The arterial blood sugar was in every experiment higher after the meal than in the fasting state; the rises were, however, in some instances very slight. The venous sugar always rose less than the arterial and, when the rise of the latter was minimal, the venous sugar occasionally fell so that it was lower at the 30 and 60 minute periods than in the fasting state. In every case, no matter how little the arterial sugar rose, a definite positive arterial-venous difference was established at the 30 or 60 minute interval or both. This difference was greater than the fasting difference in every experiment but one, Subject 7, 1927, to which reference has been made before. The difference varied from 6 to 50 mg. and, in all but two experiments, was maximal at the 30 or 60 minute points. The maximal difference occurred with equal frequency at the end of 30 or 60 minutes. It coincided with the highest point on the curve in seventeen of the twenty-four experiments and appeared when the curve was falling in six. It was less than 5 mg. twice at 30 minutes and four times at 60 minutes.

It is, then, possible in all normal individuals after a meal of 50 gm. of glucose or a mixed meal containing carbohydrate, to detect after 30 or 60 minutes or both a positive arterial-venous difference of significant magnitude, an expression of the activity of the tissues in removing sugar from the blood. This may, however, be overlooked if both 30 and 60 minute points are not determined.

Holst (7) states that the magnitude of the difference varies directly with the degree of hyperglycemia produced. It has already been pointed out that the experiments here reported do not measure the height of the blood sugar rise. There is, however, no definite correlation in these experiments between the degree of hyperglycemia observed and the magnitude of the arterial-venous difference.

In the ten experiments of the 1927 series the cutaneous blood sugar had returned to or below the original postabsorptive level at the end of 60 minutes twice, within 90 minutes three times, and twice within 120 minutes. In two other experiments, although it had not fallen to the original level, it had dropped below the upper limits of the normal blood sugar range. In only one experiment did it remain definitely elevated after 2 hours, Subject 7, the one in which the subject became faint, to which reference has been made above. The results of the other two series are not

dissimilar, although the studies were not carried beyond 90 minutes and, therefore, restoration of the normal was less frequently observed. Altogether seventeen of the twenty-four curves returned to normal within 90 minutes. Nineteen of the venous curves had fallen to the resting postabsorptive value in the same interval.

At the 90 minute period negative arterial-venous differences of considerable magnitude, 8 to 21 mg., were observed three times. On two of these occasions, Experiments 2 a and b, 1926, they were associated with definite arterial hypoglycemia. In the third, Experiment 6, 1927, the arterial sugar had returned within the normal fasting limits. These results suggest that the negative differences may represent a reaction to the postalimentary hypoglycemia which results from overstimulation of the processes which remove excess glucose from the blood stream.

*Effect of Insulin on Fasting (Postabsorptive) Blood Sugar of Normal Individuals.*

If insulin acts chiefly by accelerating the removal of sugar from blood by the tissues and may even, as Cori and Cori (11) have demonstrated, mobilize glycogen from the liver to the muscles by way of the blood, in which it exists as glucose, it should be possible to demonstrate a positive arterial-venous difference in the fasting individual during the development of insulin hypoglycemia. That such a difference is demonstrable is shown in Table II, which gives the effect of 10 units of insulin on the cutaneous and venous blood sugars of five presumably normal individuals in the post-absorptive state. Blood sugar values were determined at 30, 60, and 90 minutes after insulin injection.

In four of the five subjects the lowest blood sugar level was observed after 60 minutes. In DE the maximum effect occurred earlier. Three of the five presented distinct positive arterial-venous differences after either 30 or 60 minutes or both, coinciding with the minimal blood sugar level. In S no appreciable difference was detected. One cannot argue from this, however, that it did not occur between observations when the transient character of the difference in the others is taken into consideration.

The last subject, R.N, showed a most peculiar reaction. At the

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30 minute period, while the blood sugar was falling, a small negative difference, which had existed in the fasting state, became exaggerated. This is difficult to explain. At the lowest observed point, 60 minutes, just before he developed symptoms of insulin shock, a hardly significant positive difference had developed. That the tissues were capable of absorbing carbohydrate is demonstrated by the large difference which appeared after this subject had received carbohydrate to relieve the shock symptoms. At

TABLE II.

*Fasting Blood Sugar Values of Normal Individuals before and after Insulin.*  
Mg. per 100 cc.

Subject.	Before insulin.			After insulin.									Insulin.  <i>units</i>
	Arterial.	Venous.	Difference.	30 min.			60 min.			90 min.			
				Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	
K	99	96	3	82	73	10	61	50	11	71	66	5	10
S	79	77	2	69	69	0	51	48	3	59	55	4	10
DE	83	81	2	58	41	17	57	71	-14	45	66	-21	10
R	78	74	4	74	70	4	65	38	27	59	58	1	10
RN	89	93	-4	68	74	-6	54	50	4	149*	124*	25	10
Average.	85	82	3	71	63	8	59	52	7	59	61	-3	

\* At the 60 minute period the subject developed definite symptoms of insulin shock. The last blood examination was made at the end of 120 minutes, after he had received an orange, an apple, a small dose of glucose, and his regular breakfast. These values have been omitted in calculating the averages.

times he had glycosuria and showed abnormal reactions to carbohydrate on several occasions.

One subject, DE, developed large negative differences during his recovery period, suggesting again that the tissues may give up glucose to the blood in the reaction to hypoglycemia.

*Effect of Diseases of the Liver on Alimentary Hyperglycemia.*

If the sugar which enters the blood after the ingestion of glucose is removed both by the tissues and by the liver, one should expect

very definite abnormalities in the curve of alimentary glycemia of persons who have liver disease. Because such individuals partially or totally lack one of the mechanisms that reduces hyperglycemia, hepatic glycogen formation, the curve would presumably be excessively high or prolonged. On the other hand, the power

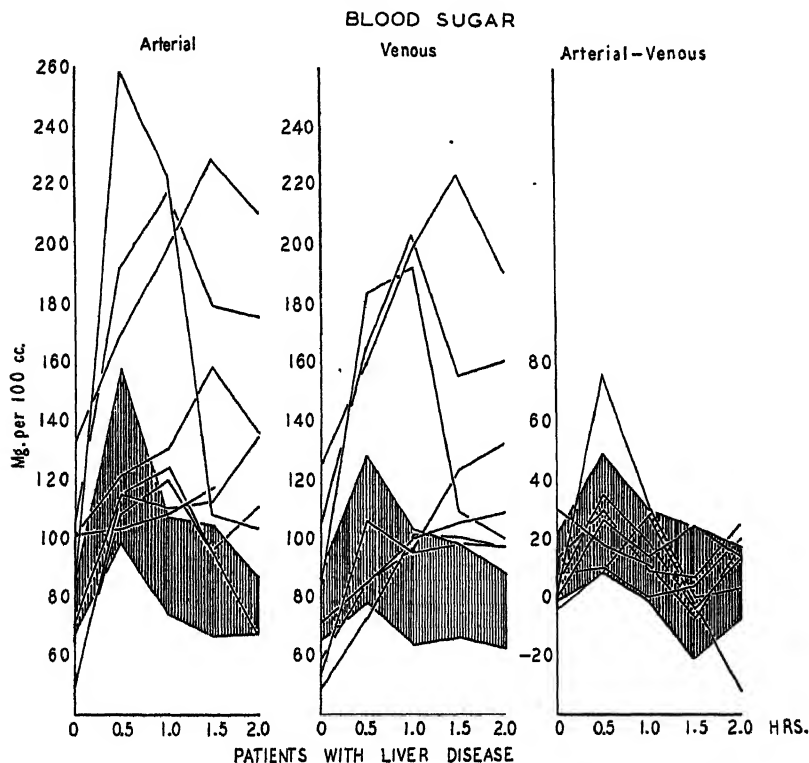


FIG. 2.

of the tissues to remove glucose remaining intact, the arterial-venous difference should manifest itself in the normal manner.

Table III and Fig. 2 show that the facts accord well with expectation. Cutaneous and venous blood sugar determinations were made before and for 2 hours after 50 gm. of glucose on seven

TABLE III.  
Blood Sugar Values in Liver Disease. Reaction to 50 Gm. of Glucose.

Mg. per 100 cc.

Subject.	Fasting.			Time after meal.												Diagnosis.
				30 min.			60 min.			90 min.			120 min.			
	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	Arterial.	Venous.	Difference.	
HM	52	48	4	108	73	35	120	101	19	96	100	-4	111	97	14	Catarrhal jaundice.
AL	49	53	-4	115	106	9	123	95	28	96	98	-2	65	97	-32	"
CP*	76	69	7	69	67	2	80	74	6	84	82	2	91	94	-3	Carcinoma of stomach with metastases to liver. Pyloric obstruction.
	71	58	13	115	85	30	109	100	9	112	105	7	134	109	25	Jaundice, before and after gastro-enterostomy.
JS	101	91	10	121	†		130			158			136			Portal cirrhosis. Ascites and jaundice.
JP	133	125	8	169	159	10	198	198	0	228	224	4	210	190	20	Portal cirrhosis. No ascites nor jaundice.
M	105	105	0	192	165	27	217	203	14	179	155	24	174	160	14	Toxic hepatitis and cholangitis.
LW	85	84	1	259	183	76	224	192	32	108	108	0	103	100	3	Arsenical hepatitis.
FM	101	71	30	103	85	18	107	96	11	116	123	-7	150	132	18	Pylephlebitis (?). Jaundice following appendectomy.
Maximum.	133	125	30	259	183	76	224	203	32	228	224	4	174	160	24	
Minimum.	49	48	-4	103	73	2	109	95	0	96	98	-7	65	97	-32	
Average.	85	78	7	159	122	30	157	141	18	134	130	4	134	126	9	

\* The failure to develop hyperglycemia in this case is probably due to non-absorption of glucose from the gastrointestinal tract.

patients with diseases affecting the liver. From the fourth, JS, only a cutaneous curve was secured because venous blood could not be obtained. Three of the eight blood sugar curves rose higher than any of those of normal subjects. The excessive hyperglycemia was evident in both cutaneous and venous bloods. Furthermore, seven of the eight arterial curves and all of the venous curves at the end of 2 hours lay above the normal range defined by the comparable experiments of 1927 (see Fig. 1). In the figure the limits of variations of nine of the normal curves (the curve of Subject 8, who almost fainted, is omitted) of the 1927 series are indicated by the shaded areas.<sup>1</sup> The curves of the subjects with liver disease are indicated by the superimposed lines. In spite of the abnormally high and prolonged hyperglycemia, in every case a very distinct positive arterial-venous difference was demonstrated, usually maximal at the 30 minute period.

It is, therefore, possible in patients with severe liver damage to demonstrate in the curve of alimentary glycemia impairment of hepatic glycogen formation without disturbance of the process of sugar removal by the tissues. The deviation from the normal in some of these cases is so small and the range of normal variation so large, that it is doubtful whether the determination of such curves will aid in the diagnosis of hepatic disease.

The first study on CP in Table III is really irrelevant to the present study. At the time when it was obtained the patient had almost complete pyloric obstruction which was relieved by gastroenterostomy before the second study was made. The first curve illustrates the small and delayed hyperglycemia that results when absorption of sugar from the alimentary tract is greatly retarded or prevented.

#### SUMMARY.

Cutaneous and venous blood sugar values have been determined simultaneously before and at half hour intervals after the ingestion of 50 gm. of glucose or a mixed breakfast containing from 75 to 100 gm. of carbohydrate in normal subjects and patients with

<sup>1</sup> These curves only are employed because they are the only normal curves that were prolonged to the 2 hour point. Both the liver curves and this normal series were determined by the same individual, MF.

diseases of the liver. Similar determinations on normal individuals in the postabsorptive state have been made before and after the administration of 10 units of insulin.

The characteristics of the normal arterial (cutaneous) and venous blood sugar after 50 gm. of glucose or a mixed breakfast are indistinguishable and can be summarized as follows:

1. In the postabsorptive state there is usually little or no difference between cutaneous and venous sugars; but positive (arterial > venous) differences of moderate degree occur with reasonable frequency, and negative differences of significant magnitude are occasionally observed.

2. At 30 minutes a definite arterial hyperglycemia is established, which usually still exists at the end of an hour. It is usually higher at 30 than at 60 minutes. In two-thirds of the experiments the cutaneous blood sugar had returned within the normal fasting range at the end of 90 minutes.

3. The venous sugar never rose as high as the arterial. Hence a definite positive arterial-venous difference, varying in magnitude from 6 to 50 mg., was detected in every instance. This was sometimes absent at either the 30 or 60 minute interval, but never at both. It was usually maximal at the point of greatest hyperglycemia. It was found even when cutaneous or venous sugar or both showed minimal rises. It had often disappeared by the end of 90 minutes and, indeed, at this time negative differences of significant or even considerable size were sometimes observed.

Insulin (10 units) when given to a normal individual in the post-absorptive state results in hypoglycemia which is usually most marked at the 60 minute period. The appearance of a distinct and significant positive arterial-venous difference can usually be demonstrated. During the recovery definite negative differences were demonstrated in one subject.

In severe liver disease an excessive or prolonged hyperglycemia can usually be demonstrated after 50 gm. of glucose. All the subjects investigated, however, developed a definite positive arterial-venous difference.

The bearing of these observations on clinical and physiological problems has been discussed briefly.

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# PROTOCOLS.

*H. M.*—Male, married, age 38. 6 weeks earlier patient had abdominal distress and clay-colored stools for a short period; 3 weeks after this a short course of fever. Jaundice commenced 2 weeks before the sugar curve was made and was beginning to clear at the time the latter was made, but was still distinctly evident. Van den Bergh and bromsulfalein tests were within normal limits. No glycosuria before or during test. *Diagnosis*: Catarrhal jaundice.

*A. L.*—Male, brass worker, age 29. Gastrointestinal symptoms of 2 weeks duration with moderate jaundice which had developed a few days later. Van den Bergh test = 14 units; bromsulfalein = 40 per cent retained at the end of 30 minutes. No glycosuria before or during test. *Diagnosis*: Catarrhal jaundice.

*C. P.*—Male, age 70. Gastrointestinal symptoms of 6 weeks duration; jaundice and high colored urine for 2 weeks. At the time of the first study the patient had definite symptoms of pyloric obstruction. Van den Bergh



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test = 22 units; bromsulfalein = 25 per cent retained at the end of 30 minutes. Glycosuria before, but none after test. The second study was made 3 weeks after an operation at which carcinoma of the stomach with pyloric obstruction and extensive involvement of liver, gall bladder, and bile ducts was found and a gastroenterostomy performed. Jaundice persisted. Van den Bergh test = 14 units. No glycosuria before or after test. *Diagnosis:* Carcinoma of stomach with metastases to liver and biliary obstruction.

*J. S.*—Male, age 62. Ascites of 2 weeks duration and slight jaundice. Van den Bergh test = 7.5 units; bromsulfalein = 42 per cent retained at the end of 30 minutes. No glycosuria before or after test. *Diagnosis:* Portal cirrhosis of the liver.

*J. B.*—Male, age 63. Hypertension and arteriosclerotic heart disease without heart failure. Large liver. Bromsulfalein = 12 per cent retained at the end of 43 minutes. No glycosuria before or after test.

*M.*—Male, age 28. Gastrointestinal symptoms and hematemesis of 10 days duration. Marked jaundice and clay-colored stools. Liver moderately enlarged. Van den Bergh test = 25 units; bromsulfalein = 45 per cent retained at the end of 30 minutes. No glycosuria before or after test. *Diagnosis:* Toxic hepatitis and cholangitis.

*L. W.*—Male, age 39. Jaundice and gastrointestinal symptoms had developed in the course of intravenous salvarsan treatment. Van den Bergh test = 11 units; bromsulfalein = 22 per cent retained at the end of 30 minutes. No glycosuria before or after test. *Diagnosis:* Arsenical hepatitis.

*F. M.*—Male, age 32. Developed septic temperature and moderate jaundice after appendectomy. On a number of occasions slight glycosuria had been observed, although no sugar was found in the urine before or after the sugar curve was made. *Diagnosis:* Pylephlebitis (?).

## DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION.

### XIX. DOES COPPER SUPPLEMENT VITAMIN B\* FOR LACTATION?†

BY BARNETT SURE.

WITH THE TECHNICAL ASSISTANCE OF DOROTHY J. WALKER,  
(From the Laboratory of Agricultural Chemistry, University of Arkansas,  
Fayetteville.)

AND THE COOPERATION OF E. H. STUART.  
(From the Research Laboratories of Eli Lilly and Company, Indianapolis.)

(Received for publication, August 27, 1928.)

In pursuing further the problem of the vitamin B requirements for lactation it has occurred to us that, since the yeast concentrates employed by us as sources of vitamin B are abundant in mineral elements, some preparations containing as much as 30 per cent ash, we may be dealing with a supplementary inorganic ion factor.

The recent discovery of Hart and coworkers (1) that copper can act as a supplement to iron in the synthesis of hemoglobin and that this element is probably the active constituent of the ash of lettuce leaves, stimulated us to investigate the possibility of copper being a limiting factor in synthetic rations used in studying vitamin B requirements for lactation. Attempts, however, to replace some of our yeast extracts with the ash equivalent to the original product resulted in absolute failure. The ash of the vitamin B concentrates was administered to nursing young as well as to lactating mothers separately from the ration, but without any response. Of course, the next step would have been to feed a

\* In this paper the term "vitamin B" is used to signify a combination of the thermolabile (antineuritic) and thermostable factors, both of which are necessary for growth.

† Research paper No. 68, Journal Series, University of Arkansas. Aided by a grant from Eli Lilly and Company, Indianapolis.

*Lactation Record of Female 4772 (First Litter, Six Young, Mother Given Six to Rear) Transferred from Stock Diet 1 on Date of Birth of Litter to Ration 1009.*

*Minimum Daily Vitamin B Requirement for Growth of Her Nursing Young (Yeast Concentrate P-19803\*).*

Date.	Female 4772.	Litter.	Food consumed during previous 24 hrs.	Date.	Female 4772.	Litter.	Food consumed during previous 24 hrs.
<i>July, 1927</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>Aug., 1927</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
19	282 <sup>1</sup>			7	188 <sup>6</sup>	135	20
20	242	34		8	184	140	17
21	232	39	3	9	178 <sup>7</sup>	143	20
22	240	41	12	10	185	156 <sup>15</sup>	26
23	240	46	11	11	183	166	27
24	238	54	4	12	178 <sup>8</sup>	162	23
25	218 <sup>2</sup>	62	8	13	180	164	23
26	224	69	15	14	176 <sup>9</sup>	164	22
27	222	76	13	15	182	170	28
28	210 <sup>3</sup>	79	14	16	186	176	21
29	214	90	15	17	183	178	25
30	212	98	17	18	184	183	31
31	208	106	14	19	178 <sup>10</sup>	184	24
<i>Aug.</i>				20	182	192	30
1	200	112	16	21	185 <sup>11</sup>	193	32
2	194	117	15	22	186 <sup>12</sup>	198	26
3	192	122	17	23	188	222	30
4	196	128 <sup>14</sup>	18	24	186 <sup>13</sup>	221	32
5	188 <sup>4</sup>	133	14	25	186	236	40
6	188 <sup>5</sup>	133	13	26	194	239 <sup>16</sup>	26

\* For method of preparation, see text.

<sup>1</sup> 10 mg. of Yeast Concentrate P-19803 to mother daily.

<sup>2</sup> 5 mg. to mother; 20 mg. to litter daily.

<sup>3</sup> 5 mg. to mother; 30 mg. to litter daily.

<sup>4</sup> 10 mg. to mother; 25 mg. to litter daily.

<sup>5</sup> 10 mg. to mother; 30 mg. to litter daily.

<sup>6</sup> 10 mg. to mother; 36 mg. to litter daily.

<sup>7</sup> 10 mg. to mother; 42 mg. to litter daily.

<sup>8</sup> 10 mg. to mother; 54 mg. to litter daily.

<sup>9</sup> 10 mg. to mother; 60 mg. to litter daily.

<sup>10</sup> 10 mg. to mother; 72 mg. to litter daily.

<sup>11</sup> 10 mg. to mother; 78 mg. to litter daily.

<sup>12</sup> 10 mg. to mother; 84 mg. to litter daily.

<sup>13</sup> Administered yeast concentrate, dried overnight in a phosphorus pentoxide desiccator, in same dosage distributed between mother and litter.

<sup>14</sup> Young opened their eyes.

<sup>15</sup> Young eating.

<sup>16</sup> Young weaned.

vitamin B product free from ash, but since the possibility of obtaining such a preparation seems quite remote at present, we were obliged to resort to another method of experimental procedure. The method of experimental inquiry employed consisted of the application of the method reported previously (2). Details of the method, describing a vitamin B unit for nursing young, will appear elsewhere (3), but in order to demonstrate the character of results we secured by supplementing some of our yeast concentrates with copper administrations to lactating mothers and to nursing young, one illustrative table is submitted (Table I). The modified biological procedure is as follows: Mothers transferred with their young, reduced to six in number, from our Stock Diet 1 (4) to vitamin B-deficient Ration 1009 (5) are allowed on date of birth of litters small amounts of concentrated vitamin B preparations to prevent rapid depletion of vitamin reserves. Vitamin therapy to the litters is begun on the 4th to 5th day of lactation; in other words, the prophylactic method is used and maintenance of nurslings is thus encountered later in lactation.

Table I illustrates a representative case of the increasing daily amounts of a yeast concentrate (prepared by adsorption on silica gel and released at various hydrogen ion concentrations) (6) necessary for growth of nursing young during various stages of the lactation period. It is quite apparent that from August 5 on, the nursing young of Female 4772 needed more than 6 mg. each of the yeast concentrate for continuous growth. A submaintenance level was reached between August 11 and 14 on a daily allowance of 7 to 9 mg. per nursling. The increase to a daily dosage of 10 mg. per young, or 1 additional mg. per nursling, was followed by appreciable growth, the response being obtained in 24 hours. Because of the hygroscopic nature of the concentrate (the hygroscopicity having been increased by frequent exposure to the air in the daily manipulation of it) we have found it difficult to weigh the material accurately between August 21 and 26, but it was determined by analysis that the product during that period carried 15 per cent moisture. It will be noted that the availability of 2 mg. more of the vitamin preparation to each nursling per day (August 24 to 26), made possible through a procedure of drying the hygroscopic vitamin B concentrated product in a phosphorus pentoxide desiccator, resulted in phenomenal growth.

Since our biological method, tested on over 200 lactating mothers and over 1000 nursing young, proved sensitive to the extent that definite responses could be secured in 24 to 48 hours we attempted to supplement their daily vitamin allowances with small amounts of copper. Calculated amounts of Baker's cupri-sulfate, C.P., were dissolved in distilled water and made up to volume so that each 0.1 cc. contained 0.01 mg. of copper. In determining the daily dosages we have followed the technique of Hart and coworkers (1) and have allowed 0.06 to 0.24 mg. daily

TABLE II.

*Effect of Administration of Copper (in Form of  $\text{CuSO}_4$ ) to Lactating Mothers or to Nursing Young on Growth of Nurslings.*

Experiment No.	Cu to lactating mother.		Cu to nursing young.			No. of administrations.	Response
	Female No.	Amount Cu administered once daily.	Litter of female No.	No. in litter.	Amount Cu administered once daily to each nursing.		
		mg.			mg.		
1338	5334	0.12	5325	6	0.01	3	None.
1342						3	"
1342			5335	6	0.01	3	"
1345			5340	5	0.01	3	"
1346			5343	6	0.01	2	"
					0.005	2	"
1347	5345	0.12				1	"
		0.24				2	"
1355	5363	0.12				3	"
	5364	0.12				3	"
1356	5365	0.06				3	"

dosages to the lactating mothers. To the nursing young, weighing 22 to 30 gm. each, we have allowed 0.01 mg. daily per animal in most cases (Table II), but in a few cases have reduced the daily dosage to 0.005 mg. per young. The supplementary copper administrations were given to such young that reached a maintenance level during the latter part of lactation at which time the nurslings received approximately 60 per cent of the optimum daily administration of a vitamin B concentrate necessary for continuous growth. Table II shows that twenty-three nursing

young made no response in 48 to 72 hours. The same young, however, responded readily (in 24 hours) when the daily dosage of the concentrate was increased from 15 to 20 mg. per nursing per day. A further response was obtained when the daily dosage to each young was increased to 25 mg. Five lactating mothers showed no response to administrations of 0.06 to 0.24 mg. of copper per animal per day, in addition to a daily dosage of 150 to 300 mg. of the same concentrate allowed the nursing young. The maximum daily dosage of this concentrate found necessary to wean a litter of six young was 150 mg.

We are also showing in this paper one illustration of a series of experiments completed last summer on the potency of head lettuce as a source of vitamin B for lactation.<sup>1</sup> Chart I shows clearly that 30 gm. of fresh head lettuce, administered daily, separately from the ration, to the lactating mother at the point of depletion of her vitamin reserves, is followed by complete failure in growth of the nursing young. For brevity we have deleted the daily growth record of the lactating mothers and the daily food consumption data and are showing graphically only the character of growth of the nursing young. The amount of lettuce we have furnished our animals daily was the maximum they would consume in addition to their basal diet, but no response in lactation was obtained.

If copper is essential for lactation and for the growth of nursing young, it must have been furnished by the lower levels of our vitamin B concentrate before maintenance was reached during the later stages of lactation. At least, additional increments of growth could not be secured by copper administrations in dosages which Hart and coworkers found effectual in the synthesis of hemoglobin and also for growth in rats that have become anemic on an exclusively whole milk diet. On the other hand, when the daily dosage of our vitamin concentrate was increased we never failed to receive a response in the growth of nursing young in as short an interval as 24 hours.

<sup>1</sup> The lettuce experiments were carried out with the assistance of Miss Mildred Cummings, a senior student in the Department of Home Economics, during the year 1927.

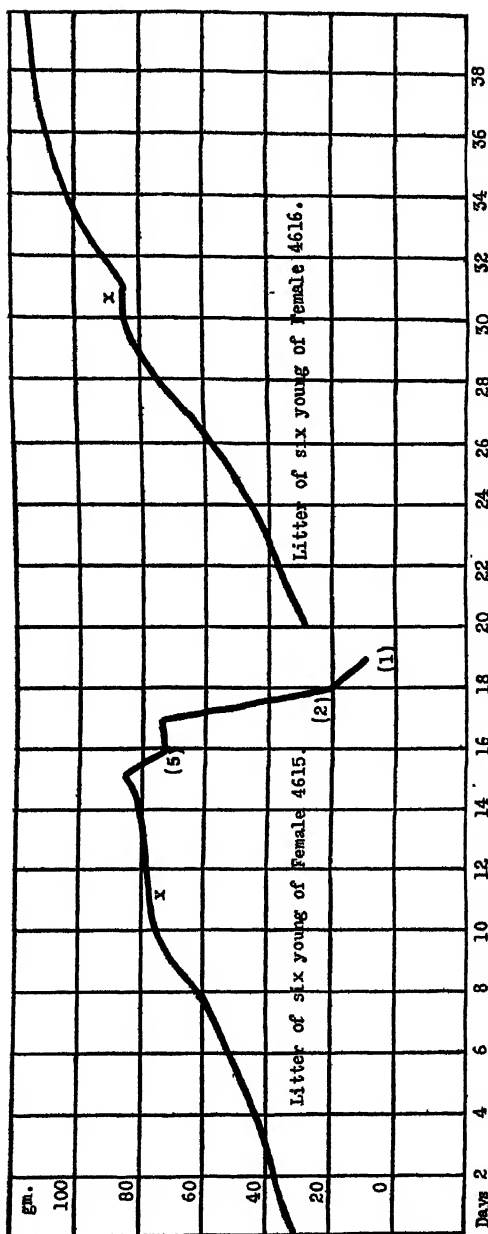


CHART I. Growth of nursing young of lactating Mothers 4615 and 4616, transferred on date of birth of litters from Stock Diet 1 to vitamin B-deficient Ration 1009. The composition of Ration 1009 is as follows: casein (purified) 20, agar-agar 2, Salts 185\* 4, butter fat 5, dextrin 69. At point x mothers were allowed 30 gm. of fresh head lettuce daily. The numbers in parentheses indicate how many live young were left in the litter of six.

\* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

## SUMMARY.

Copper was found to have no supplementary value to a vitamin B concentrate for lactation.

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## DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION.

### XX. A DIFFERENTIATION OF THE VITAMIN B COMPLEX IN RICE POLISHINGS AS EVIDENCED IN STUDIES OF LACTATION.\*

BY BARNETT SURE.

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AND THE COOPERATION OF E. H. STUART.  
(*From the Research Laboratories of Eli Lilly and Company, Indianapolis.*)  
(Received for publication, August 27, 1923.)

During the last few years considerable evidence has been presented to the effect that the dietary substance which was heretofore known as vitamin B is a complex composed of at least two components, one which is thermolabile and the other thermostable, both of which in combination are essential for growth. The literature on the subject has been recently reviewed by Sherman and Axtmayer (1) and by Chick and Roscoe (2).

In recent communications it has been reported that the lactating rat is incapable of secreting the vitamin B complex quantitatively in the milk (3). In this paper a differentiation is made of the two dietary factors associated with the vitamin B complex as found in rice polishings, and experimental evidence is submitted showing that the lactating animal is inefficient in secreting both the stable and the labile factors in the milk.

The experimental procedure was to transfer lactating mothers from our colony Stock Diet 1 (4) to vitamin B-deficient Ration 1009 (5) on the date of the birth of the litters. At the point of depletion of vitamin reserves of the mother the ration was changed, rice polishings replacing an equivalent amount of dextrin in the

\* Research paper No. 89, Journal Series, University of Arkansas. Aided by a grant from Eli Lilly and Company, Indianapolis.

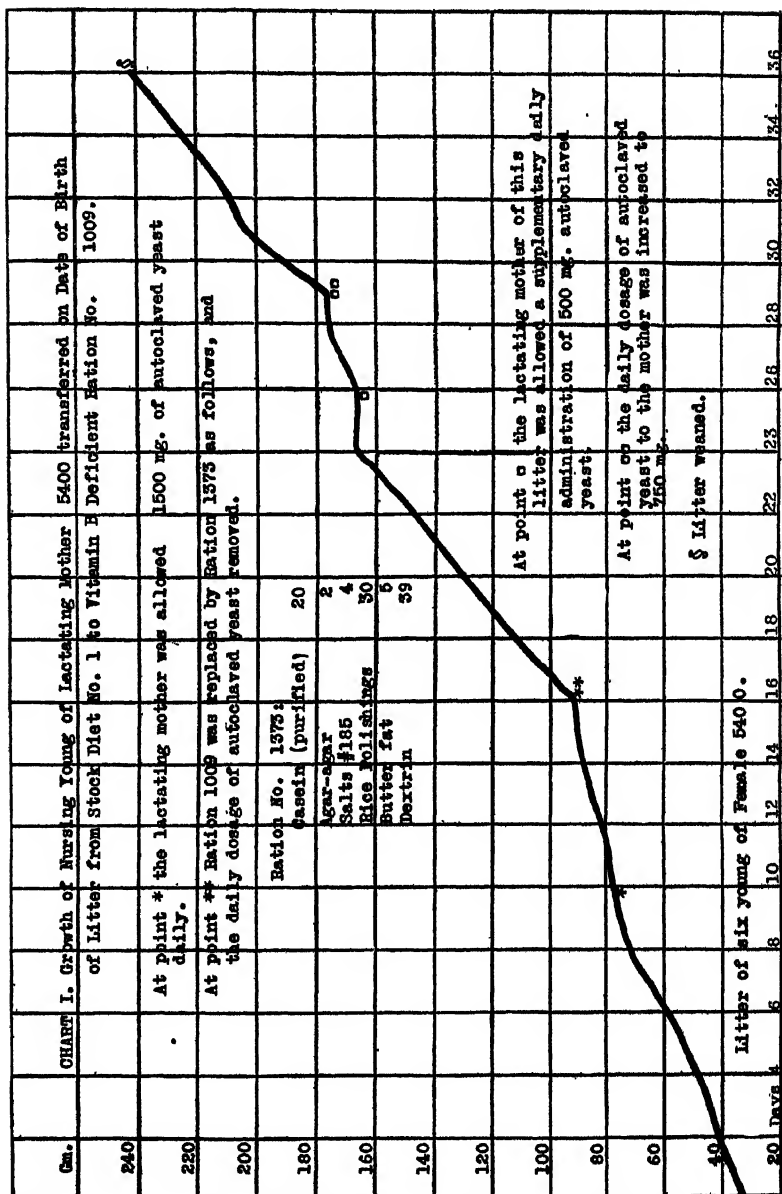


CHART I.

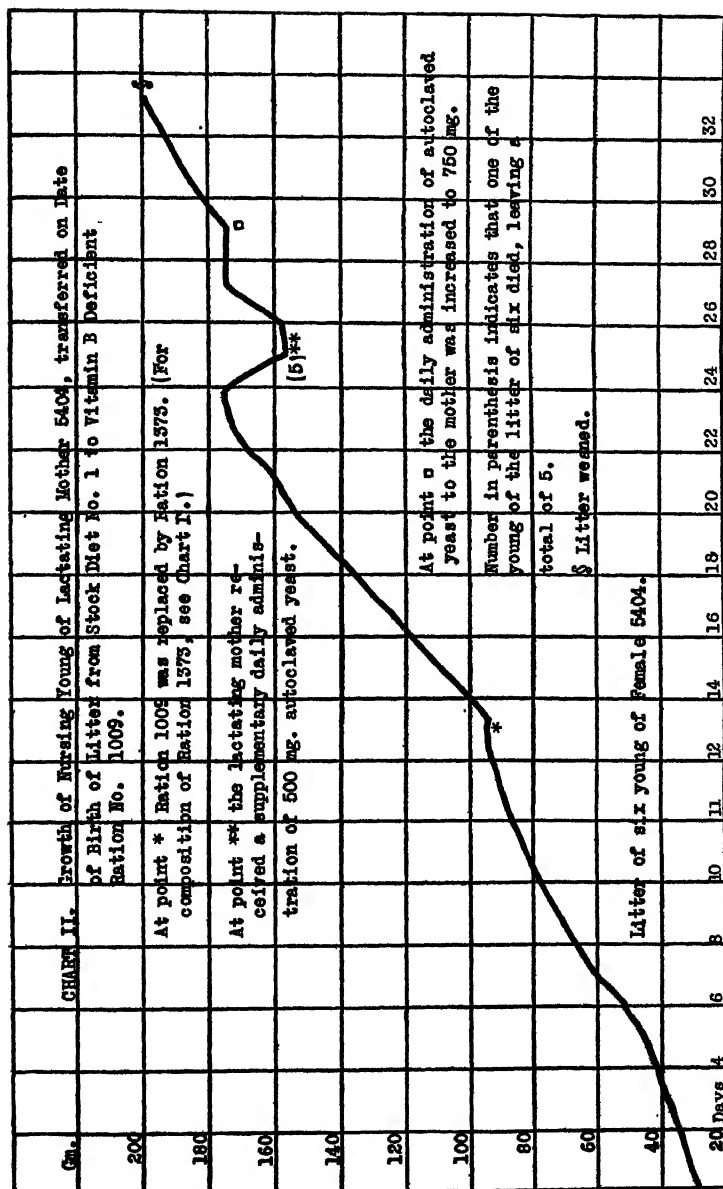


CHART II.

diet. For brevity we are deleting daily records of weight of mothers as well as daily food consumption data, and are showing graphically only representative cases of our results as evidenced by growth of nursing litters.

Chart I shows that at point \* before the ration was changed, a slight response was secured by the supplementary daily administration of 1500 mg. of yeast autoclaved for 5 hours at 15 to 18 pounds pressure, but marked response followed at point \*\* when the autoclaved yeast was removed and the ration changed, 30 per cent rice polishings replacing an equivalent amount of dextrin in the diet. Such procedure resulted in phenomenal growth in the nursing young for the subsequent 8 days, and another maintenance curve in the litter was reached on the 24th day of lactation. On the 26th day, at point □ the lactating mother received a supplementary daily allowance of 500 mg. of autoclaved yeast, which resulted in growth of the nurslings for 48 hours, but another plateau was encountered the subsequent 24 hours. On the 29th day, at point □ □ the increase of the daily dosage of autoclaved yeast to the mother to 750 mg. stimulated excellent growth in the litter for the rest of the 7 day experimental period.

Chart II shows the results of a similar experiment. In this case the point of depletion of the stable factor in the lactating mother could not be readily anticipated, and one of the six nurslings died, undoubtedly for lack of an optimum amount of the thermostable substance. Following the death of this nursing young the supplementary daily administration of autoclaved yeast to the mother was begun. The responses to the 500 and 750 mg. daily allowances of autoclaved yeast to the lactating mother, judging by the character of growth of the nursing young, are apparent.

The results shown in Charts I and II are typical of six additional experiments and show that rice polishings fed at a 30 per cent plane of intake provide an abundance of the antineuritic (labile) factor for lactation, but an insufficiency of the stable factor, also a necessary constituent of milk for continuous growth of nursing young.

Charts III and IV show that rice polishings, fed at a 20 per cent level, do not even furnish enough of the antineuritic factor for growth of the nursing young, since as much as 1500 mg. daily of autoclaved yeast failed as a supplement to the lactating mothers.

Chart IV reveals that, even when the preventive method is employed by administering the supplementary allowances of autoclaved yeast to the mother before depletion of reserves of the stable

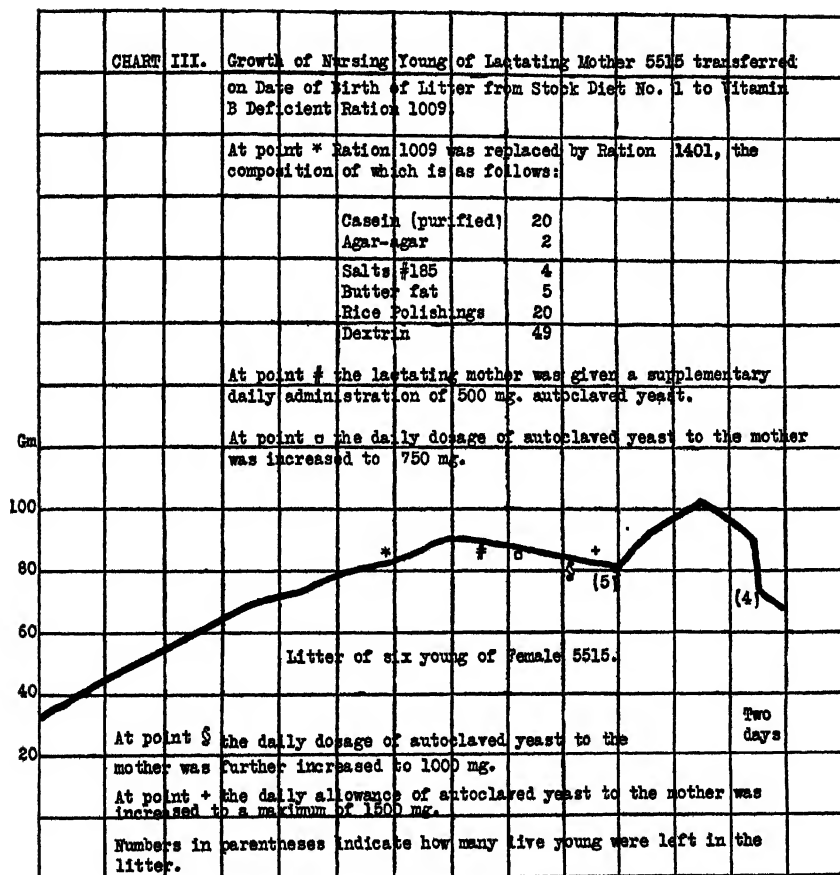


CHART III.

factor, we cannot circumvent subsequent infant mortality, because of the insufficient amounts of the antineuritic (labile) factor supplied by the lower level of rice polishings.

*Inefficiency of the Lactating Mother in Secreting Stable Factor in Milk.*

We have observed in a number of cases that, although the lactating mothers responded to the administrations of autoclaved yeast supplementing a ration containing 30 per cent rice polish-

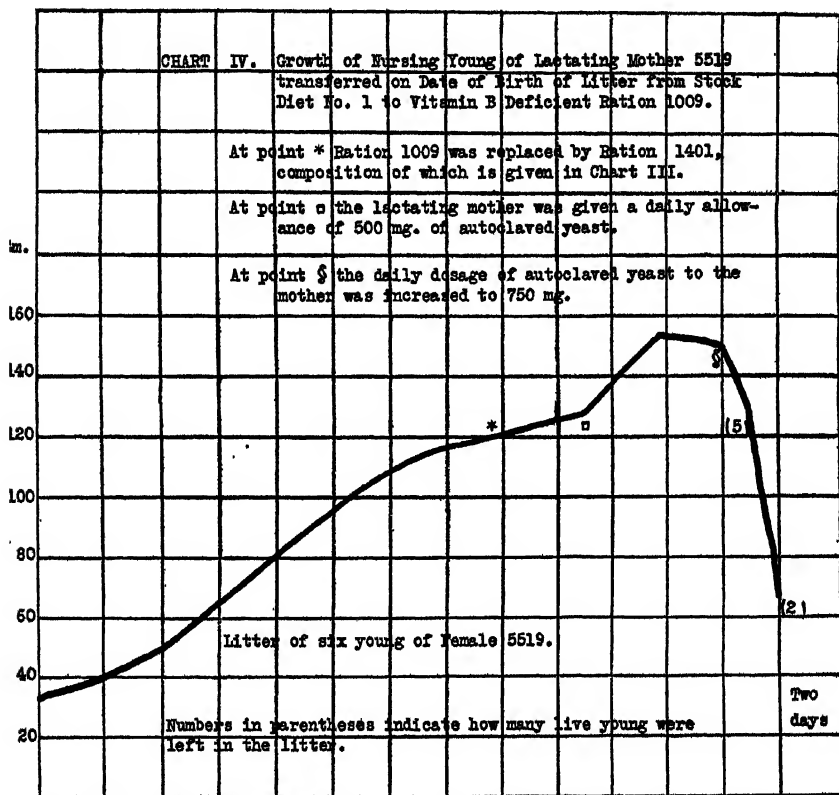


CHART IV.

ings, they were rearing their young at a subnormal rate. All attempts to distribute a daily dosage of 500 to 750 mg. between lactating mothers and nursing young resulted in failure in four litters, because the nursing young absolutely refused to consume

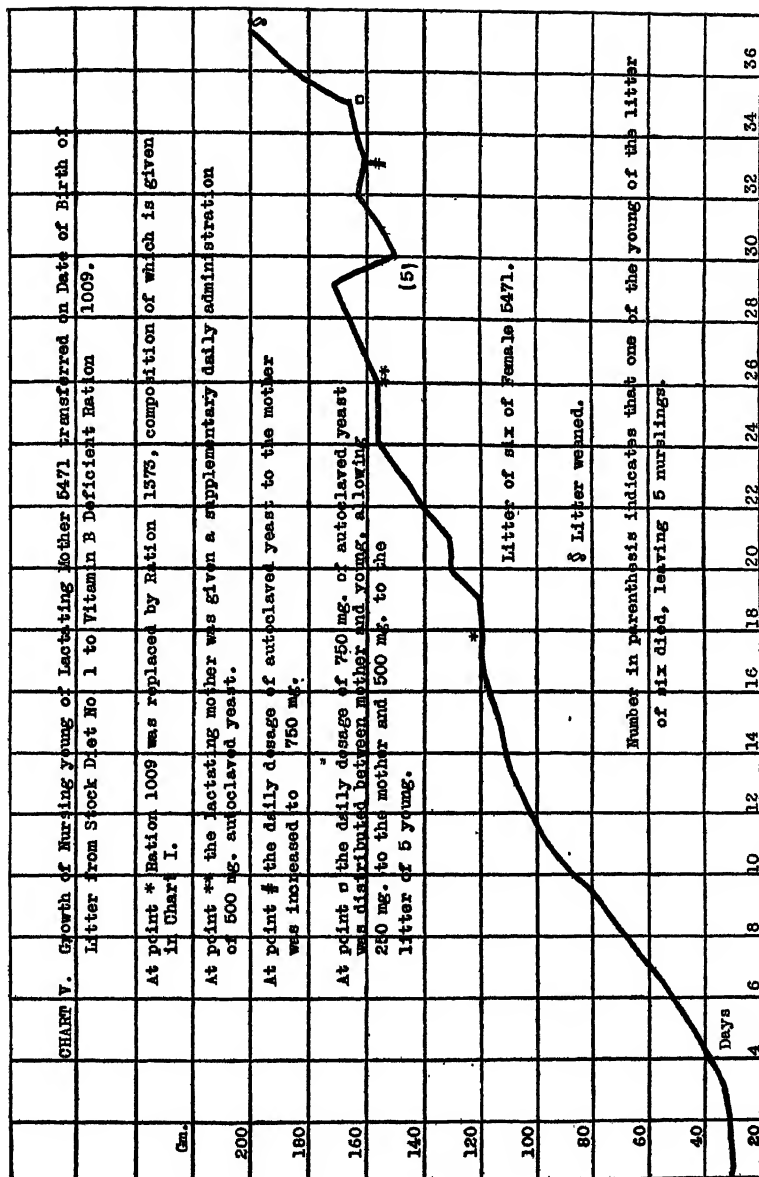


CHART V.



the autoclaved product. This we were, however, later able to accomplish with two litters. Chart V shows the results of the character of response of one of those litters. Between the 32nd and 35th day of lactation the remaining litter of five young of Female 5471 gained 5 gm. when the lactating mother was receiving a supplementary daily administration of 750 mg. of autoclaved yeast. On the 35th day of lactation the 750 mg. daily allowance of autoclaved yeast was distributed, allowing 250 mg. to the mother and 500 mg. to the litter, or 100 mg. to each nursing, and during the subsequent 48 hours the litter gained 35 gm.

Another litter responded in a similar manner when during the latter part of lactation the 500 mg. daily dosage of autoclaved yeast, allowed the lactating mother, was given to her litter.

In addition to the two experiments just cited, we have obtained identical results with four more litters on a somewhat modified dietary régime. In these cases the mother and young received a distribution of a vitamin concentrate prepared from an alcoholic extract of rice polishings by adsorption on fullers' earth. In this way we have provided an abundance of the antineuritic factor to the mother and nursing young. The mothers received in addition a daily dosage of 1500 mg. of autoclaved yeast. When, during the later part of lactation the litters showed prolonged maintenance, the daily portion of autoclaved yeast given the mother was distributed, allowing the nursing young the greater part of the dosage. In every case such procedure was followed by accelerated growth of the litters. From the above experiments we conclude that the lactating rat is incapable of secreting the stable, growth-promoting factor, associated with the vitamin B complex, quantitatively in the milk.

*Inefficiency of the Lactating Mother in Secreting Antineuritic (Labile) Factor in Milk.*

In proceeding with the quantitative study of the secretion of the antineuritic (labile) factor in the milk we took advantage of the fact that in the preparation of various vitamin concentrates we had access to one product obtained from yeast which was deficient in the antineuritic but abundant in the stable factor. We also had an alcoholic extract from rice polishings which, although unusually potent in the antineuritic, was markedly deficient in the

stable substance. After a number of litters reached a maintenance curve during the later part of lactation on a distribution of 130 mg. daily of our Yeast Concentrate 21 (abundant in the stable, but deficient in the antineuritic factor) between mother and young, allowing daily 10 mg. to the mother and 120 mg. to litters of six young, each mother received in addition 6 drops of a concentrated alcoholic extract of rice polishings. The responses in 48 hours were insignificant. The litters either continued to maintain their weight or gained 2 to 3 gm. The 6 drops of the same vitamin concentrate were then administered to the litters, 1 drop to each young daily, and in every case the young responded phenomenally. During the subsequent 2 days each litter gained as much as 30 gm. Six litters of 48 young were subjected to this experimental study and not one of these nurslings failed to respond readily to supplementary administrations of the antineuritic factor furnished by the alcoholic extract of rice polishings, whereas all the mothers failed to secrete this substance in the milk in sufficient amounts to meet the requirements of the nursing young for growth. It is apparent, then, from these experiments that the lactating rat is unable to secrete quantitatively in the milk the antineuritic (labile) growth-promoting factor as well as the stable, growth-promoting substance.

#### DISCUSSION.

From our food consumption records it has become apparent that 7 gm. of rice polishings per lactating rat per day furnish an abundance of the antineuritic (labile) factor, but an inadequate amount of the stable factor for lactation. On the other hand, 2.3 gm. of rice polishings per animal per day have supplied an abundance of both factors in rats for growth to the extent that young animals (whose stores of vitamin reserves of both factors had been entirely depleted when the experiments were begun at weaning) trebled their weaning weights in 58 days. At this writing we have based such findings on twenty-four animals. It is clear, then, that the requirements of the stable factor for lactation are more than 3 times that necessary for growth. The requirements of the antineuritic (labile) substance for lactation are also considerably greater than for growth, since we have found that a daily allowance of 5 gm. of rice polishings (as a source of the antineuritic

factor) per lactating rat per day proved inadequate for milk secretion, whereas a daily amount of 2.3 gm. of rice polishings per animal per day has so far been excellent for growth for as long as 2 months. We are continuing these growth experiments for longer periods of time.

Our findings are not in harmony with the recent report of Evans and Burr (6) that "lactation delinquency is due to inadequate antineuritic vitamin, and in no degree to inadequate growth-promoting vitamin B," since our conclusions are that larger amounts of both factors are essential for lactation than for growth. The references of Evans and Burr (6) and Hunt (7) to the antineuritic as contrasted with the growth-promoting substance are misleading, since both the antineuritic and stable factors are growth-promoting. The responses to either of these dietary factors, associated with the vitamin B complex, in growth of nursing young have been demonstrated in the experimental evidence presented in this communication.

*Nomenclature of Dietary Factors Associated with Vitamin B Complex.*

A differentiation of the fat-soluble vitamins A and D in cod liver oil was made by McCollum and coworkers (8), mainly on the grounds of thermostability. We are, therefore, justified from the extensive evidence recently accumulated that the dietary complex, which McCollum and Kennedy in 1915 termed water-soluble B (9) is also made up of two specific vitamins. The recent work of Chick and Roscoe (2) confirms the original observations of Goldberger and associates (10) that a deficiency of the vitamin, which is thermostable, produces in the rat a dermatitis quite similar to that observed in human pellagra. It also now seems apparent that Eijkman, when in 1897 he produced the classical demonstration of polyneuritis in animals (11), was dealing with a deficiency of the antineuritic, thermolabile vitamin.

The English biochemists have provisionally adopted the nomenclature B<sub>1</sub> and B<sub>2</sub> for the labile and stable vitamins respectively (2). Such nomenclature is objectionable because it still leaves the impression that we are dealing with two phases of one rather than with two separate vitamins. Sherman and Axtmayer (1) have, therefore, suggested the adoption of letters F and G to

represent the labile and stable vitamins respectively, and that the term vitamin B should represent the combination of vitamins F and G. The objection to this nomenclature is that no special physiological function is assigned to Eijkman's classical anti-beriberi vitamin, which has for the last 13 years been known as vitamin B, and the universal recognition of which has been largely responsible for the stimulation of vitamin research. I am, therefore, in accord with the recent suggestion of Hogan and Hunter (12) that we retain the letter B for the thermolabile, anti-beriberi vitamin of Eijkman, and add the letter F to represent the thermostable, anti-pellagra vitamin of Goldberger and associates, with the understanding that both of these vitamins are growth-promoting in the sense that fat-soluble vitamins A and D are both growth-promoting.

#### SUMMARY.

In accordance with the nomenclature suggested in the text for the dietary factors associated with the vitamin B complex, it is concluded from the results reported in this paper that rice polishings contain in addition to large amounts of vitamin B an appreciable amount of vitamin F for growth, but from the standpoint of lactation rice polishings, while abundant in vitamin B, proved deficient in vitamin F.

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R. T. P.

## STUDIES OF THE INHIBITORY ACTION OF AN EXTRACT OF PANCREAS UPON GLYCOLYSIS.

### I. EFFECT OF PANCREATIC INHIBITOR ON THE GLYCOLYSIS OF MUSCLE TISSUE AND MUSCLE EXTRACT.

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(Received for publication, August 9, 1928.)

In 1915, Winfield and Hopkins (20) reported the discovery of a substance in the pancreas which, they believed, exerted an inhibitory effect upon the glycolytic activity of muscle. Foster and Woodrow (9) found that this inhibitor checked to about 60 per cent the glycolysis of muscle but did not entirely prevent it. Foster's (8) explanation of this partial inhibition of lactic acid formation in muscle seemed inadequate and without convincing experimental proof. We, therefore, undertook an analysis of the effect of this substance on muscle and other tissues known to have glycolytic activity. Shortly before these studies were commenced, Warburg's (18) important experiments on the glycolytic properties of cancer cells had been reported. The relation which glycolysis might have to the whole subject of growth seemed to render more important any agent which might check the glycolytic activity of tissue. These experiments were undertaken, therefore, with the idea not only of determining the nature and effects of the pancreatic factor of Winfield and Hopkins but also as a method of study and differentiation of the glycolytic mechanism of tissues.

The present paper is concerned with the preparation of the pancreatic factor and its effect upon muscle tissue and upon the active muscle extract of Meyerhof (13). A brief preliminary report of a portion of the work was presented before the Society of Clinical Investigation in 1927 (1). Other aspects were briefly discussed by Ronzoni (15).

*Analytical Methods.*

For lactic acid and free reducing substance the proteins were removed by precipitation with  $\text{HgCl}_2$  in 2 per cent  $\text{HCl}$ . The filtrates were neutralized with  $\text{NaOH}$  to pH 6.8 after removal of  $\text{Hg}$  with  $\text{H}_2\text{S}$  and removal of  $\text{H}_2\text{S}$  by aeration. Somogyi's (17) modification of the Shaffer-Hartmann method was used for sugar determination. Lactic acid was determined after precipitation with  $\text{CuSO}_4$  and  $\text{Ca}(\text{OH})_2$  by Clausen's permanganate method as modified by Friedemann, Cotonio, and Shaffer (10).

Total carbohydrate was determined as glucose after hydrolysis of the muscle or muscle extract with 0.5 N  $\text{HCl}$  for 3 hours, neutralization to pH 6.8 with  $\text{NaOH}$ , and precipitation with  $\text{Hg}(\text{NO}_3)_2$ ,  $\text{NaOH}$  being used to precipitate  $\text{HgO}$ . After complete removal of  $\text{Hg}$  by  $\text{H}_2\text{S}$  and removal of  $\text{H}_2\text{S}$  by aeration, the filtrates were neutralized to pH 6.8. This procedure has been shown to remove neither lactic acid nor sugar from solution (Ronconi and Wallen-Lawrence (16)). The values for sugar by this method are somewhat higher than the true sugar but since the reducing non-sugar does not increase on incubation, the differences in carbohydrate before and after incubation should represent the true loss of carbohydrate. After removal of sugar, lactic acid was determined on these filtrates. The results are somewhat lower than those obtained from  $\text{HgCl}_2$  filtrates and, since added lactic acid can be completely recovered, probably represent more nearly the true values. The phosphates were determined in  $\text{HgCl}_2$  filtrates by Briggs' (2) modification of the Bell-Doisy method. The acidity of the phosphate standard was always adjusted to equal that of the filtrate. The labile phosphate of muscle observed by Fiske and Subbarow (7) and by Eggleton and Eggleton (4) does not enter into the values reported here since it was included in the determination of the initial free phosphate as well as in the samples incubated for 2 hours.

Manometric determinations of acid production were made with Warburg's (19) modification of the Barcroft manometer. The retention factor for lactic acid was determined by Negelein's (14) method of measuring the  $\text{CO}_2$  which can be liberated by a known amount of lactic acid from the buffer system which, in our experiments, consisted of muscle extract with added phosphate and bicarbonate. The theoretical value calculated from the

amount of lactic acid was divided by the amount of  $\text{CO}_2$  actually liberated, the quotient being used as the retention factor. The observed values of  $\text{CO}_2$  multiplied by this factor gave the corrected values for the  $\text{CO}_2$  which was calculated as equivalent to the acid formed.

*Preparation of the Pancreatic Factor.*

In early experiments the pancreatic factor was prepared according to Foster and Woodrow's (9) directions. After two failures attributable, we believed, to the fact that we were unable to control the temperatures at which dehydration was carried on but which we later found to be due to the relative insolubility of the factor in 70 per cent alcohol, we succeeded in producing an extract possessing about one-half the activity of that described by Foster. Her method proving tedious because of lack of facilities for keeping the temperature below  $0^\circ$ , we substituted the following procedure.

External fat was removed from thoroughly chilled fresh pig pancreas, which was then finely ground through a meat chopper. To this were added 2 volumes of distilled water chilled to  $0^\circ$ . The mixture was kept at  $0^\circ$  and frequently shaken for a period of 2 hours, then strained through gauze (Filtrate A). The residue was again mixed with 2 volumes of water, was frozen to rupture any unplasmolyzed cells, and kept at  $0^\circ$  overnight. The next morning it was thoroughly shaken and filtered through gauze. The residue was washed once with water (Filtrate B). A third extraction contained but a trace of inhibiting substance and was discarded.

To Filtrate A 2 volumes of 95 per cent alcohol were added at once. After standing for 1 hour this was poured on a filter and placed in the refrigerator overnight. By morning the entire amount had filtered. Filtrate B was treated in the same way. Since it had about the same activity as the first extraction, the two were combined. The alcohol was removed by evaporation at  $30^\circ$  with a current of warm air blown across the surface. The residue was diluted with water to a convenient volume and centrifuged. A layer of fat which separated at the surface was removed and the remainder extracted 3 times with ether. The ether was removed by aeration and the remainder filtered. Continuous liquid extraction removes the fat more completely with but slight effect



on the activity. Since the small amount of fat which still remained in the extract seemed to have no effect on its activity, continuous extraction was ordinarily omitted. The extract was standardized and concentrated to such a volume that 1 cc. would inhibit the activity of 10 cc. of muscle extract. To preserve the extract 2 volumes of cold 95 per cent alcohol were added. This caused a slight precipitation of protein and salts. Since this usually carried with it some of the active material, it was not removed.

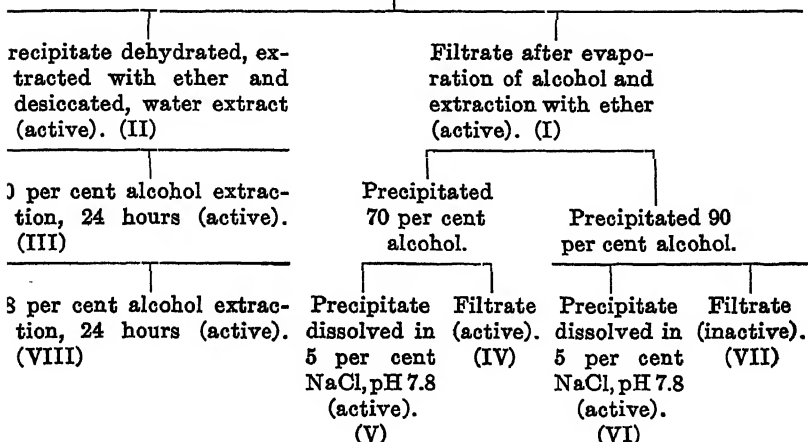
An extract prepared in this way can be kept as long as 3 months at 0° without loss of activity. For use, it was concentrated to its original volume at 30°. The remainder of the alcohol was removed by being extracted twice with ether and the ether was removed by aeration. This extract has a pH of about 5.0 to 5.2, so, before use, it was neutralized to the pH of the buffer mixture to which it was to be added. As would be expected from its high phosphate content, it has a fairly high buffering quality, especially when neutralized to pH 7.8. The extract may be sterilized by Berkefeld filtration which does not diminish its activity.

If the original water extract of the pancreas was precipitated with 70 per cent alcohol the protein precipitation was more complete but there was also a great loss of activity of the extract. If, however, an active extract, made as described above and containing only a small amount of protein, was precipitated with 70 per cent alcohol, the precipitation of protein was slight and the loss of activity was correspondingly less. The precipitation of a larger amount of protein adsorbs more of the active substance. On the other hand, 90 per cent alcohol precipitates nearly all of the inhibiting factor from a water extract even when it contains only a small amount of nitrogenous material.

Since precipitation of proteins reduced the activity of the water extract, it was thought probable that the original precipitation with 58 per cent alcohol had removed some of the active substance. The precipitate from the first two treatments with alcohol were therefore combined, dehydrated with alcohol, extracted with ether, and dried in a desiccator. There resulted a white hygroscopic powder, a water extract of which was found to have, per gm. of dried pancreas, at least as great an activity as had the alcoholic filtrate. Its tryptic activity, however, was somewhat greater, so, for the experiments here reported, the alcoholic extract was employed.

The accompanying diagram presents the facts concerning the inhibitory activity of the various fractions.

Pancreas extracted twice with water precipitated with 2 volumes of 95 per cent alcohol.



In Table I there is a summary of the inhibitory, amylolytic, and tryptic activities of the fractions. The inhibitory activity is expressed in terms of the number of gm. of the original wet pancreas which were necessary to inhibit the activity in 10 cc. of muscle extract. Owing to the difficulty of accurate standardization these quantities are only approximate, but they give a fair idea of the potency of each fraction. The amylolytic and tryptic activity are expressed respectively in percentages of total carbohydrate from glycogen and total nitrogen from casein, rendered reducing or soluble in 2 hours by the action of the amount of pancreatic substance indicated in the first column. A correction was made for the soluble nitrogen and reducing substance present in the pancreatic extract. These values were determined on  $\text{HgCl}_2$  filtrates. The total increase in reducing substances is not due entirely to the complete formation of glucose. The degradation of glycogen gives rise not only to glucose but also to intermediate degradation products which have reducing properties. The relative amounts of these various products will be considered in a later paper.

To test further the solubility of the inhibiting substance in alcohol, dry pancreas powder was prepared according to Foster and extracted in the following ways. The activity of the extracted portions was tested on the muscle extract described by Meyerhof (13). Its activity was tested by measuring the inhibition which it exerted on the loss of phosphates from muscle extract

TABLE I.

Fraction No.	Inhibitory action.*	Amylolytic activity.*	Proteolytic activity.*
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
I	0.200	29.2	12.2
II	0.100	61.5	23.2
III	2.00	13.0	1.6
IV	1.00	10.9	1.8
V	0.50	30.2	10.8
VI	0.50	29.8	11.2
VII	Inactive.		
VIII	0.50	28.2	3.2

\* Expressed in terms of the number of gm. of the original wet pancreas necessary to inhibit the activity of 10 cc. of muscle extract.

TABLE II.

Preparation of fraction.	Equivalent in terms of dried pancreas.	Amount used.
		<i>cc.</i>
I. Extracted with 100 cc. water 2 hrs....	1 cc. = 50 mg.	2
II. " " 50 " 58 per cent alcohol 2 hrs. ....	1 " = 100 "	5
III. Extracted with 50 cc. 70 per cent alcohol 2 hrs. ....	1 " = 100 "	5
IV. Filtrate after water extraction and precipitation with 70 per cent alcohol.	1 " = 100 "	5
V. Precipitate of Fraction IV in 50 cc. water. ....	1 " = 100 "	5

treated with sodium fluoride and glycogen rather than by the less accurate method of measuring its inhibitory action on lactic acid production.

For each extraction 5.0 gm. of pancreas powder were employed. The various filtrates were prepared and used as shown in Table II.

From Table III it appears that extraction for 2 hours with 58

or 70 per cent alcohol (Fraction II or III) removes but little of the inhibiting factors and that precipitation of the water extract with 70 per cent alcohol removes most of the inhibiting factor (Fraction IV). This has either been destroyed or rendered insoluble in water since it is not present in the water extract of this

TABLE III.

Fraction No.	Activity of pancreatic factor.			Analysis of pancreatic factor.			
	Inhibition.	Amylo-lytic activity.*	Proteo-lytic activity.*	Non-protein N.	Reduction value.	Free phosphate.	Total N.
	per cent	mg.	mg.	mg.	mg.	mg.	mg.
I	100	51.1	8.1	3.1	1.9	0.06	8.02
II	30	14.8	1.0	1.0	0.7	0.11	1.7
III	21	14.2	1.0	1.0	0.7	0.14	1.5
IV	20	10.9	1.2	4.4	1.8	0.16	4.59
V	10	4.8	0.0	0.9	1.8	0.34	0.44

\* The amylolytic activity is measured in mg. of free reducing substance expressed as glucose which appears as the result of the action of this substance on muscle extract containing 60 mg. of glycogen. The proteolytic activity is expressed in mg. increase in nitrogen appearing in the  $\text{HgCl}_2$  filtrate of muscle under the same conditions.

TABLE IV.

Preparation of fraction.	Equivalent in terms of dry pancreas.	Amount used.
	mg.	cc.
I. Extract in 100 cc. water.....	50	1
II. Alcohol precipitate extracted with 50 cc. water....	500	5
III. Insoluble fraction of above dissolved in NaCl pH 7.8.....	100	2
IV. 50 cc. of filtrate from alcohol precipitate.....	500	5
V. Extracted in 50 cc. 70 per cent alcohol.....	500	5
VI. " " 50 " 58 " " " .....	200	2

precipitate (Fraction V). Much of the protein also has been rendered insoluble as is shown by the total nitrogen figures of Fraction V. It seemed probable that the inhibiting substance as well as the proteolytic and amylolytic enzymes remained adsorbed on the insoluble protein.

In the following experiment designed to test this question, water extracts were prepared as in the preceding experiment. The portion of the extract precipitated by 70 per cent alcohol was first extracted well with water. The insoluble portion was separated by centrifugation. The supernatant fluid was removed and was designated Extract II. The remaining precipitate was dissolved with 0.5 per cent NaCl at a pH 7.8. Most of it went into solution, leaving but a small residue which was discarded. This formed Extract III. Since 2 hours extraction had proved ineffective in extraction of the inhibiting factor with alcohol, extraction for 24 hours at refrigerator temperature was tried.

TABLE V.  
Activity of pancreatic factor expressed as in Table III.

Fraction No.	Activity of pancreatic factor.			Analysis of pancreatic factor.			
	Inhibition.	Amylo-lytic.	Proteo-lytic.	Non-protein N.	Total N.	Free phosphate.	Reducing value.
	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
I	100.0	14.4	1.8	4.5	8.1	0.08	0.32
II	38.0	2.7	1.6	0.3	1.0	Trace.	0.45
III	100.0	9.5	3.9	0.2	2.1	"	0.50
IV	32.0	6.5	1.5	5.1	5.1	0.31	1.0
V	22.5	2.4	0.6	3.6	3.6	0.25	0.7
VI	100.0	11.0	2.4	4.14	4.3	0.10	0.9

The preparations given in Table IV were employed. In each instance 5.0 gm. of pancreas powder were used. It will be seen in Table V that the water extract is distinctly more potent than the other extracts. 50 mg. will produce complete inhibition in 10 cc. of muscle extract and this amount may have been a great excess. The portion precipitated by 70 per cent alcohol is relatively insoluble in water but is soluble in 0.5 per cent NaCl at a pH of 7.8. The inhibiting factor is only slightly soluble in 70 per cent alcohol and is almost completely removed by precipitating an active, protein-containing watery extract with this concentration of alcohol. It is, however, more soluble in 58 per cent alcohol, such extracts having about one-fourth the activity of the water extract. It should be pointed out that all extracts having a low inhibitory activity also have a slight proteolytic and amylolytic effect. It is

a well known fact that these enzymes are adsorbed by protein. The fact that all three are removed by the same procedure in no way proves, however, that the inhibitory effect is due to either the amylolytic or tryptic enzyme.

According to the preceding observations, it requires more than 500 mg. of dry pancreas, when extracted with 70 per cent alcohol, to inhibit the activity of 10 cc. of muscle extract. A 58 per cent alcohol extract of 200 mg. will completely inhibit the action of 10 cc. of muscle extract and this may be an excess. 50 mg. extracted with water have a similar effect. It is interesting to note that although per gm. of dried pancreas the water extract is about 4 times as potent as the 58 per cent alcohol extract, it is per gm. of total solids only about one-fiftieth as active. An extract made by precipitating a water extract with 58 per cent alcohol contains about one-half the activity of the original water extract.

#### *Effect of Inhibiting Factor on Muscle.*

After producing an active substance by precipitating the water extract with 58 per cent alcohol, we were able to confirm Foster and Woodrow's (9) observations as to its effect on rabbit muscle. In all the experiments, the conditions were similar. The muscle was removed from the animal under amytal anesthesia and frozen as rapidly as possible in a freezing mixture of ice and salt. This required about 40 minutes. The muscle was then ground through a cold meat chopper and thoroughly mixed and sampled while frozen. These results are duplicates on two samples of muscle only. After being weighed, the muscle was placed in flasks containing 5 cc. of 0.15 M  $\text{NaH}_2\text{PO}_4$  solution with the inhibitor or an equivalent amount of Ringer's solution plus 1 cc. of 0.01 M KCN and was immersed in an ice bath. All samples were allowed to remain in this condition for half an hour. Enough 0.15 M NaOH was added to bring the solution to a pH 7.8.

To obtain the original values for lactic acid and carbohydrate, control samples were used. To each of these 10 cc. of cold 2 per cent HCl were added immediately. The flasks were placed in a freezing mixture for 20 minutes when 10 cc. of  $\text{HgCl}_2$  were added; other controls to show the amount of lactic acid which the muscle could produce were incubated with the flasks containing the inhibitor for 2 hours at 37.5°. They were then treated with

HgCl<sub>2</sub> as above. The results are shown in Table VI. It was evident from these early experiments that after the action of the inhibitor, HgCl<sub>2</sub> was incapable of precipitating the proteins completely. As a consequence, although carbohydrate determination showed small changes and usually a slight increase, the results were not considered satisfactory and are not included in Table VI.

It will be seen that the inhibition in lactic acid formation was between 38 and 68 per cent. It appears from these results that although there was a considerable variation in the percentage change, the amount of lactic acid produced in the presence of the inhibitor was of remarkably constant value. On the other hand, the total lactic acid that the uninhibited muscle was capable of producing, varied with different animals and with the conditions

TABLE VI.

Initial value.	Lactic acid, mg. per 100 gm.				Per cent inhibition.
	Without inhibitor.		With inhibitor.		
	After incubation.	Increase.	After incubation.	Increase.	
203	689	486	410	207	57.4
120	731	611	318	198	67.8
132	743	611	363	231	62.2
105	792	687	394	289	58.0
89	722	633	335	246	61.2
63	683	620	288	225	63.5
230	620	390	470	240	38.5

under which it was removed. In no case was inhibition complete. This is in agreement with the experiment of Foster and Woodrow (9) who to explain it suggested two mechanisms for the production of lactic acid. Since this suggestion seemed to introduce an unnecessary complication, for which there was no direct evidence, we sought a simpler explanation. The most obvious possibility was a failure of penetration of the active substance into the muscle tissue.

It was believed that, if insufficient permeation of the pancreatic factor were responsible, the length of time during which the inhibitor was exposed to the muscle would influence the degree of inhibition. To study this it was desirable to expose the muscle to the inhibitor for varying periods before the glycolysis of the muscle

commenced. Furthermore, it was necessary to find some substance which would temporarily check glycolysis but which could be neutralized whenever desired. For this purpose we selected  $\text{NaH}_2\text{PO}_4$  which at low temperatures will prevent almost completely the production of lactic acid. For a long time it does not damage the activity of the muscle as is shown by the fact that neutralization causes a complete resumption of function. The behavior of muscle when treated with sodium acid phosphate is

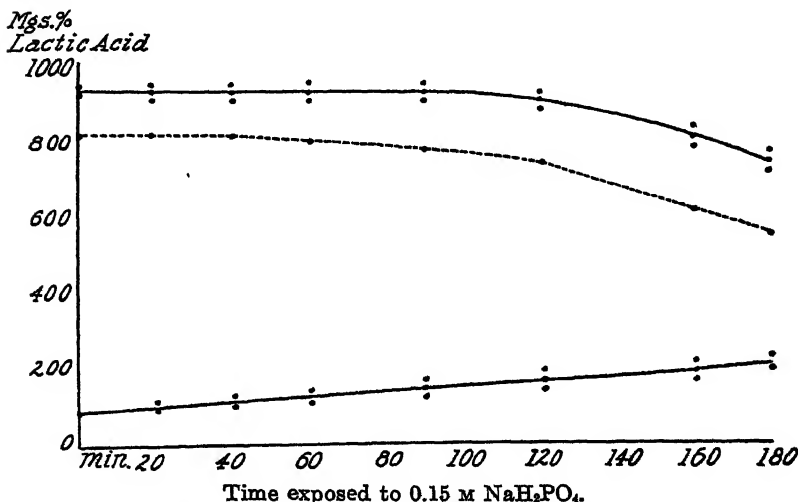


CHART I. Effect of  $\text{NaH}_2\text{PO}_4$  at 4 to 6° on lactic acid content and on ability of muscle to produce lactic acid after neutralization. Lower solid line, lactic acid content at time of neutralization. Upper solid line, lactic acid content 2 hours after neutralization. Broken line, lactic acid production during 2 hours at 37.5° following neutralization.

represented in Chart I. The lower solid line represents the lactic acid content of the muscle before neutralization. The upper solid line represents the lactic acid content after neutralization and after incubation for 2 hours at 37.5°. The dash line represents the difference or the amount of lactic acid produced during the period of incubation. It will be seen that treatment of the muscle with  $\text{NaH}_2\text{PO}_4$  for 2 hours causes no deterioration in its later glycolytic activity.

Having determined that the glycolysis of muscle could be con-



trolled by  $\text{NaH}_2\text{PO}_4$ , we performed the following experiments. Several samples of the same muscle together with the inhibitor were treated in acid phosphate solution. After varying intervals of time, the acid phosphate was neutralized. Simultaneous observations similar to those in Chart I were employed as controls. Of these, one set was used to give the initial lactic acid at the time of neutralization and the other to show the ability of the muscle to produce lactic acid. The results may be found in Chart II in which the percentage of inhibition of lactic acid formation is charted against the time the muscle has been in contact with the

% inhibition

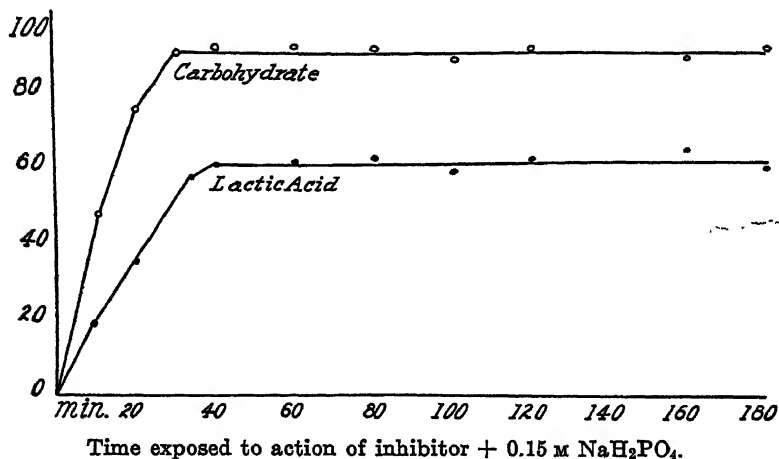


CHART II. Inhibition of carbohydrate and lactic acid changes, showing effect of duration of exposure of inhibiting factor.

inhibitor. It will be seen that the maximum inhibition occurs after about  $\frac{1}{2}$  hour of contact of the inhibitor with the finely hashed muscle. Longer contact does not increase the percentage of inhibition although this maximum may vary with different muscles. It would appear from this that the failure of complete inhibition is not due to insufficient permeation. The experiments do not exclude the possibility that the pancreatic substance is adsorbed by some material other than the one on which it acts. Preliminary experiments indicate that the incomplete inhibition may be explained by the presence of a precursor of lactic acid which is not

glycogen and the breakdown of which involves phosphate liberation as well as the formation of lactic acid. This tends to support the work of Embden and Hayman (5).

A comparison of carbohydrate loss and of lactic acid formation in muscle with and without the inhibiting substance showed a much more pronounced effect on the loss of carbohydrate than upon the production of lactic acid. There was almost no loss of carbohydrate as determined by acid hydrolysis and subsequent determination of reducing substance. The following experiments will make this point clear.

Since errors in sampling muscle are inevitable and as much as 10 or 12 per cent variation in lactic acid and carbohydrate content of different samples of the same muscle may result, three or four separate analyses were made in each case. The results of four such determinations with four different pancreatic extracts are reported. Frozen rabbit muscle was used in these experiments. 4 gm. samples were weighed into flasks containing 5 cc. of 0.15 M  $\text{NaH}_2\text{PO}_4$ . Eight flasks were used as controls to determine the values in muscle as sampled. Eight were incubated to obtain the normal reaction of the muscle and eight were incubated with the inhibitor. All were allowed to stand for 45 minutes in the cold. Those to be incubated were then neutralized. To the controls were added 10  $\mu\text{c}$ . of N HCl. These were allowed to remain for half an hour below  $0^\circ$ . Of these, four were hydrolyzed for total carbohydrate. To four were added 10 cc. of  $\text{HgCl}_2$ . After 2 hours incubation the remaining samples were treated in the same manner.

In Table VII it is clearly shown that the inhibition in the formation of lactic acid is between 40 and 60 per cent and that the amount of lactic acid produced in the presence of the inhibitor is not accompanied by a corresponding loss in carbohydrate. It is also striking that the amount of lactic acid produced after inhibition is a fairly constant quantity, though different samples of uninhibited muscle show a considerable variation in the amounts they are capable of producing.

It should be noted also that in all of the experiments upon uninhibited muscle the carbohydrate loss is not as great as the production of lactic acid. This implies a precursor of lactic acid that yields little if any reducing substance on acid hydrolysis.

Presumably this is hexosephosphate. If in these experiments all of this is broken down to lactic acid, it must be in amount equivalent to approximately 150 to 200 mg. of lactic acid. The discrepancy between carbohydrate loss and lactic acid formation is not apparent when glycogen is added to the reaction mixture. This may indicate that with high carbohydrate concentration, the formation of an intermediary substance keeps pace with or even exceeds its breakdown.

*Action of Inhibitor on Muscle Extract.*

The extraction of the lactic acid-producing enzymes from muscle by Meyerhof (13) has opened the way for a more careful investiga-

TABLE VII.

*Effect of Inhibiting Factor on Lactic Acid Production, Carbohydrate Loss, and Increase in Free Sugar Expressed in Mg. Per 100 Gm. of Muscle.*

Inhibitor No.	Without inhibitor.			With inhibitor.			Per cent inhibition.	
	Lactic acid.	Total carbohydrate.	Free sugar.	Lactic acid.	Total carbohydrate.	Free sugar.	Lactic acid.	Total carbohydrate.
III	+671	-490	+24	+245	-31	+67	63.5	93.6
IV	+601	-491	+69	+238	-34	+132	60.3	93.0
V	+316	-259	+76	+203	-11	+144	35.7	95.8
V*	+593	-641	+96	+213	-39	+201	64.1	94.1
VI	+480	-393	+48	+275	-31	+123	42.7	93.5
VI†	+590	-612	+63	+185	-36	+183	68.6	93.8

\* Same as Inhibitor V with added glycogen.

† Same as Inhibitor VI with added glycogen.

tion of the effect of the inhibiting factor with a technique which eliminates the errors inherent in the sampling of muscle.

Muscle extract was prepared from rabbit muscle. The animal was anesthetized with amytal and bled to death. The muscles were removed as quickly as possible and dropped into water at 0°. After thorough chilling they were ground through a cold grinder and returned to the flasks with sufficient water to make by volume a 1:3 dilution of the muscle. The flasks were placed in an ice and salt bath at 2° and allowed to extract for half an hour. The tissues were filtered through gauze under considerable pressure. Phosphate was then added in the form of Na<sub>2</sub>HPO<sub>4</sub>, mixed

thoroughly, making about a 1:4 dilution of the original muscle. It was then filtered through fine muslin to remove any remaining tissue. Sodium bicarbonate was added to a concentration of 0.04 M. Samples of 10 cc. each were measured into 100 cc. graduated flasks. To control samples was added HCl, one set for hydrolysis and one for precipitation by  $\text{HgCl}_2$  which was added at once. After this the samples were placed in the refrigerator.

TABLE VIII.

	Muscle extract and glycogen.			Muscle extract and inhibitor and glycogen.		
	Before.	After.	Change.	Before.	After.	Change.
Inhibitor IV. Dec. 12, 1927.						
Lactic acid.....	13.10	32.5	+19.4	14.0	14.0	± 0.0
Total carbohydrate.....	62.0	37.8	-24.2	73.0	72.0	-1.0
Free sugar.....	3.0	12.4	-9.4	5.2	34.6	+29.40
Non-protein N.....	9.3	10.6	+1.3	10.9	18.9	+8.00
Phosphate.....	8.72	5.0	-3.72	9.6	9.62	+0.02
Inhibitor V. Dec. 15, 1927.						
Lactic acid.....	11.4	37.4	+26.0	12.80	12.8	±0.0
Total carbohydrate.....	72.3	37.9	-34.4	72.9	72.9	±0.0
Free sugar.....	6.1	11.4	+5.3	7.1	22.4	+15.3
Non-protein N.....	9.6	10.0	+0.4	10.6	25.4	+14.8
Phosphate.....	9.10	5.53	-3.57	9.34	9.5	+0.16
Inhibitor VI. Feb. 3, 1928.						
Lactic acid.....	7.2	19.15	+11.9	8.0	10.2	+2.2
Total carbohydrate.....	60.8	42.6	-18.2	63.8	60.1	-3.7
Inhibitor VII. June 4, 1928.						
Lactic acid.....	9.6	23.7	+14.1	11.9	11.9	±0.0
Total carbohydrate.....	63.8	40.4	-23.4	73.0	73.4	+0.4
Free sugar.....	7.0	16.2	+9.2	7.0	22.0	+15.0
Phosphate.....	10.76	8.77	-1.93	10.76	10.78	±0.0

On muscle extract the action of the inhibitor is almost instantaneous, so glycogen was added immediately after the inhibitor was introduced. Since the inhibitor contains a slight amount of reducing substance and some sulfide-binding substance, it was necessary to run controls with the inhibitor added. It was thought that the proteolytic activity might yield some reducing substances and other substances that would affect the determina-

tion of lactic acid. Controls were run without the addition of glycogen, the inhibitor having acted on the extract a similar length of time. In all cases the reaction was allowed to continue for 2 hours after the addition of glycogen.

Four experiments reported in Table VIII show the action of four different pancreatic preparations on muscle extract. It will be seen that the pancreatic factor inhibits completely the produc-

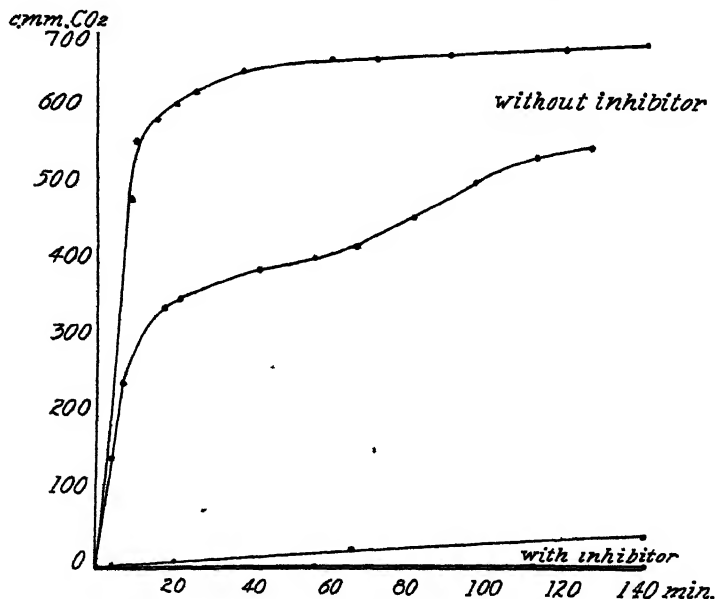


CHART. III. Action of pancreatic inhibition on the glycolysis of muscle extract.

tion of lactic acid and also the loss of carbohydrate and of phosphate. Both the free reducing substance and the non-protein nitrogen content are increased. The latter, although due to proteolytic action, does not indicate that the inhibition is due to tissue digestion. Foster (8) has demonstrated that the inhibiting action of the pancreatic factor is not due to trypsin. The complete inhibition of carbohydrate and phosphate loss suggest that the action may be one which prevents the esterification of phosphate with carbohydrate, a probable step in the formation of lactic acid by muscle.

In contradistinction to muscle, the loss of carbohydrate in uninhibited muscle extract is greater than the lactic acid which is formed. This suggests that the precursor which gives rise to the extra lactic acid in muscle is not extracted or is broken down during the process of extraction. In the muscle extract, a coincident loss of phosphate confirms the observations of Davenport and Cotonio (3) and of Meyerhof (13) and suggests the probable formation of a hexosephosphate ester during the course of the experiment.

TABLE IX.

*Action of Pancreatic Factor on Muscle Extract in Presence of NaF Changes Occurring in 2 Hours in 10 Cc. of Extract Plus 60 Mg. of Glycogen Incubated at 28°.*

Inhibitor No.	Without inhibitor.			With inhibitor.		
	Phosphate.	Total carbo- hydrate.	Free sugar.	Phosphate.	Total carbo- hydrate.	Free sugar.
	mg.	mg.	mg.	mg.	mg.	mg.
3	-5.6	-22.8	+4.8	+0.21	±0.00	+22.1
	-7.2	-34.0	+5.9	+0.23	+0.12	+25.3
4	-6.3	-28.6	+4.6	+0.12	+0.09	+20.2
5	-4.9	-17.6	+8.2	+0.29	+0.21	+12.6
	-8.3	-34.1	+7.4	+0.18	+0.18	+15.9
6	-6.8	-28.2	+6.2	+0.09	±0.00	+11.3
	-7.3	-35.5	+5.1	+0.13	+0.09	+10.9
	-4.9	-21.6	+8.6	±0.00	+0.11	+11.6
	-8.2	-29.6	+7.6	+0.15	±0.00	+12.5

In addition to the chemical experiments, observations were made with the manometric method of Warburg (18) upon the action of the inhibitor on muscle extract. Two of the experiments are represented in Chart III.

The data obtained by the manometric method are uncorrected for phosphate changes. The correction applied by Meyerhof (13) does not make the lactic acid calculated from the CO<sub>2</sub> check with that chemically determined. Either some other factor is active or the factors applied are not correct. Certainly with the liberation of considerable quantities of acid the buffering capacity of

the solution would change during the activity. Since the retention factor was determined in extracts in which no lactic acid has accumulated, this may be a source of considerable error in the calculation. The curves in Chart III are included only to show the immediate action of the inhibiting substance upon glycolysis.

The effect of the pancreatic factor was further tested in the presence of sodium fluoride. While this paper was being written work of a similar nature confirming our results was published by

TABLE X.

*Formation of Lactic Acid from Preformed Ester in Presence of Inhibiting Factor (Mg. in 10 Cc.).*

Conditions.	Phosphate.		Lactic acid.		Total carbohydrate.		Free sugar.	
	Present.	Change.	Present.	Change.	Present.	Change.	Present.	Change.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Part I. Extract as made.....	14.30		10.20		36.9		2.8	
" II. 15 min. later..	12.36	-1.94	14.98	+4.78	24.3	-12.6	3.0	+0.2
Samples treated 2 hrs. as follows. Changes based on amount present at 15 min.								
Part III. Nothing added.	11.30	-1.06	21.70	+6.72	12.3	-12.0	5.8	+2.8
" IV. Inhibitor added.....	14.80	+2.50	18.30	+3.32	23.5	-1.8	17.0	+14.0
" V. NaF added.....	8.80	-3.56	14.76	-0.22	13.5	-10.8	7.6	+4.6
" VI. " and inhibitor added.....	12.20	-0.16	14.82	-0.16	23.9	-0.4	18.5	+15.5

McCullagh (12). The results are included in Table IX. Here, as before, we note a complete inhibition of phosphate disappearance and of carbohydrate loss when the production of lactic acid has been inhibited by sodium fluoride. Again there are increases in free sugar and small increases in free phosphate.

From these experiments it is obvious that the pancreatic factor inhibits the esterification of phosphate and carbohydrate, but there is no evidence that this is its only mode of action. Meyerhof (13) has shown that there is a loss of phosphate from muscle extract

even when the formation of lactic acid is uninhibited by sodium fluoride. This is interpreted as evidence that the esterification mechanism is more efficient than the mechanism by which it is split into lactic acid and free phosphate. Davenport and Cotonio (3) have confirmed this observation and show some quantitative relationships between the excess loss of carbohydrate over the lactic acid formed and the phosphate lost during the same interval. Our own data show this to be true. By utilizing the very rapid phosphate changes which occur immediately after glycogen is added to muscle extract, it was thought possible to accumulate hexosephosphate, to inhibit its further formation by addition of the inhibiting factor, and to follow its breakdown by determina-

TABLE XI.

*Formation of Lactic Acid from Preformed Ester in Presence of Inhibiting Factor (Mg. in 10 Cc.).*

Extract No.	Loss of phosphate in 15 min.	Changes occurring in following 2 hrs.		
		With inhibitor.		With inhibitor + NaF.
		Phosphate.	Lactic acid.	Phosphate.
1	1.43	+1.53	+2.89	-0.10
2	1.14	1.16	2.49	-0.11
3	0.03	0.09	0.19	+0.07
4	0.00	0.06	0.13	-0.06
5	1.08	1.09	1.98	-0.09

tion of the increase in lactic acid and free phosphate. If successful, this experiment would prove that there was no effect on the breakdown of the ester. To test this, glycogen was added to muscle extract and a sample (Part I) precipitated at once to get the initial value. After 15 minutes the remainder was divided into five parts: Part II precipitated, Part III nothing added, Part IV inhibitor added, Part V NaF added, Part VI NaF and inhibitor added. Parts III, IV, V, and VI were precipitated after 2 hours. The data are tabulated in Table X. At the end of 15 minutes 1.94 mg. of phosphate had disappeared. 2 hours later all of this has been liberated even in the presence of the inhibiting factor. With its liberation there is also an increase in the lactic acid content. That the liberated phosphate is greater than that which had



disappeared is due to the usual slight increase of phosphate in the presence of the inhibiting substance. That the formation of hexosephosphate was inhibited is shown by Part VI in which there was a negligible decrease in phosphate after the inhibitor was added. The slight change might indicate that the action of the inhibitor is not quite instantaneous. The experiment of Table III shows definitely that the action of the inhibiting factor influences only the synthesis of hexosephosphate and does not influence the breakdown of the preformed ester.

The experiment was repeated several times. All muscle extracts do not show the ability to accumulate hexosephosphate while the lactic acid mechanism is still active and uninhibited by sodium fluoride. In three out of five subsequent experiments there was a measurable loss of phosphate during the first 15 minutes and this was liberated after the addition of the inhibiting substance with the formation of lactic acid. That the inhibitor acts with great rapidity is shown by the slight changes when it is added with sodium fluoride. These results are given in Table XI.

#### DISCUSSION.

At present the quantitative relationships between the changes in carbohydrate, free phosphate, and lactic acid are rendered uncertain because the reducing value of varying amounts of hexosephosphate is unknown. Furthermore, the effect of acid hydrolysis on the reducing value of this substance has not been accurately determined. We have recently perfected a method by which hexosephosphate can be removed completely from solution without interference with either lactic acid or glucose. The more exact quantitative relationships between these changes is the subject of a later communication.

We have shown that the action of the inhibiting factor prevents loss of carbohydrate and of phosphate in muscle extract. Foster's (8) observation that the inhibition of lactic acid production was incomplete in muscle is confirmed with the added observation that the disappearance of carbohydrate is also almost completely inhibited. The production of a relatively constant amount of lactic acid with but minor changes in hydrolyzable reducing substances is indicative of another precursor of lactic acid. The increase in free phosphate coincident with the

increase of lactic acid suggests that this substance is the lactacidogen of Embden (6) and that it does exist preformed in muscle. Lohman's (11) recent observations, however, indicating that this increase in phosphate in muscle incubated in  $\text{NaH}_2\text{CO}_3$  is from pyrophosphate and not from hexosephosphate, render this interpretation of the data questionable and the nature of this precursor of lactic acid in muscle still undetermined.

In muscle extract which contains none of this precursor it is shown that when phosphate and carbohydrate changes are completely inhibited by the pancreatic factor there is no production of lactic acid, but in so far as these remain uninhibited, lactic acid formation is unaffected. This is in agreement with the observations on muscle. Also it is shown that small amounts of the phosphate-carbohydrate complex which are accumulated in muscle extract without the addition of NaF and which are measured by a loss of carbohydrate and phosphate, are subsequently split into lactic acid and phosphate after complete inhibition of esterifying mechanisms by the pancreatic factor.

In muscle extract then the only mechanism by which lactic acid is produced is through the intermediary involving carbohydrate and phosphate disappearance, presumably hexosephosphate. Unless the mechanism existing in muscle, frozen and hashed, is different from that in muscle extract, this is evidence that the preformed precursor of lactic acid existing in muscle is a complex involving carbohydrate and phosphate.

Although the physiological action of this substance has not been investigated and no inferences can be drawn as to its biological significance, it should be of importance in studying the mechanism of glycolysis in tissue other than in muscle. As NaF inhibits the formation of lactic acid from some intermediary, so this substance inhibits the esterification of phosphates and carbohydrates and without otherwise interfering with the formation of lactic acid.

#### CONCLUSION.

1. In confirmation of the work of Winfield and Hopkins, it is found that from the pancreas an extract may be prepared which exerts an inhibitory effect upon the glycolysis of muscle. A simplification in its preparation has been devised and its solubility in different concentrations of alcohol has been studied.

2. By means of an adequate amount of this extract the glycolysis of muscle may be inhibited approximately 60 per cent. Larger amounts or stronger extracts do not increase the inhibition. The failure to inhibit the glycolysis completely is not due to incomplete permeation of the extract into the muscle. The inhibition of carbohydrate breakdown is greater than the inhibition in the formation of lactic acid, indicating an origin of lactic acid from some substance other than carbohydrate.

3. The pancreatic factor has been shown to inhibit the disappearance of phosphate and carbohydrate from muscle extract. This inhibition is also complete in the presence of NaF which apparently stimulates the esterification of phosphate and carbohydrate as well as inhibits the formation of lactic acid. In so far as this precursor of lactic acid is preformed or its production only partially inhibited, lactic acid production is uninfluenced.

4. Evidence is given that the mechanism by which muscle extract forms lactic acid involves the disappearance from solution of both phosphate and carbohydrate, presumably with the formation of hexosephosphate.

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# STUDIES OF THE INHIBITORY ACTION OF AN EXTRACT OF PANCREAS UPON GLYCOLYSIS.

## II. EFFECT OF THE INHIBITOR UPON THE GLYCOLYSIS OF MALIGNANT TUMORS.

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The experiments of Warburg, Posener, and Negelein (10) established the important fact that the surviving tissue of malignant tumors has an unusually high glycolytic activity. A few months later, a report was published by Foster and Woodrow (2) who had renewed the work of Winfield and Hopkins (11) on a pancreatic extract which had the peculiar power of inhibiting the glycolysis of muscle. Since it appeared that the glycolytic activity of cancer might be associated in some way with its rapid growth, any substance which could materially influence this property assumed an added importance. One of the original incentives which induced us to undertake these studies of the pancreatic factor was to determine whether its effect upon the glycolysis of malignant growths was similar to its observed action on muscle. The results of the work on muscle and the mechanism by which the pancreatic factor inhibits glycolysis have been published in Paper I of this series (6). In this communication we report the effect of the pancreatic inhibitor upon the glycolysis of malignant tumors, together with some studies of the special peculiarities of glycolytic activity in cancer cells.

### *Methods and Materials.*

Glycolysis was measured by the method described by Warburg (9). Cancer tissue, cut in slices of sufficient thinness, were introduced into a Ringer's bicarbonate solution having a bicarbonate concentration of 0.025 M and containing for the usual observa-

tions 0.2 per cent of glucose. In some experiments a phosphate buffer solution of 0.15 M having a pH of 7.8 replaced Ringer's solution.

Vessels modified from those employed by Warburg (10) were used in all experiments. Before the start of the observation, tissue and solutions were thoroughly saturated with gas mixtures which contained always 5 per cent carbon dioxide and, according to the particular necessities of the experiment, oxygen, air, or nitrogen. The reaction mixture and tissue were incubated in a water bath at 37.5° for 1 to 3 hours. The tissue was then quickly rinsed in distilled water, placed on weighing glasses, thoroughly dried, and weighed. To determine the effect of the pancreatic substance, identical preparation was made except that the inhibitor in the desired amount replaced a part of the Ringer's solution. Inasmuch as the pancreatic extract contained some buffering substances, it was necessary to introduce a retention factor into the calculations whenever the inhibitor was added to the reaction mixture. This was determined by Negelein's (5) method of measuring the carbon dioxide, which can be liberated from the reaction mixture by a known amount of lactic acid, and comparing it with the amount theoretically produced by the reaction between sodium bicarbonate and the same amount of lactic acid. From the experimental data, calculation was made of the quantity of carbon dioxide which was liberated because of the formation of lactic acid in the reaction mixture. This was expressed, after Warburg, as  $Q_{CO_2}$ , the quantity of carbon dioxide per mg. of tissue per hour.

In many of the experiments, the glycolytic activity of the tissue was determined by chemical analysis. Since the lactic acid production is high in tumors, chemical determinations may be made on small amounts of tissue. When such analyses were to be made, the slices of tumor tissue were allowed to float in Ringer's solution at 37.5° until the completion of slicing, which usually consumed 30 to 45 minutes after the animal was killed. They were then distributed into flasks containing 5 cc. of Ringer's bicarbonate solution containing 0.25 per cent glucose, which Warburg found to be the optimum concentration for the production of lactic acid. These were then incubated at 37.5° for a period of 2 or 3 hours, after which the protein was precipitated by

Schenk's method, making a total volume of 50 cc. After the mercury was removed with  $\text{H}_2\text{S}$ , sugar was determined by the Shaffer-Hartmann (7) method. In the later experiments Somogyi's (8) modified solution was used. After the removal of interfering substances by the Salkowski-Van Slyke procedure, lactic acid determinations were made with the permanganate oxidation as modified by Friedemann, Cotonio, and Shaffer (3) and Clausen's method of titration. Phosphate values were obtained by the Briggs' (1) modification of the Bell-Doisy method.

The determination of the amount of tissue was difficult. To weigh the fresh tissue was impossible since it carried considerable amounts of Ringer's solution. Removing and drying the tissue after the reaction had taken place introduced errors in the chemical determination, owing to the loss involved in the procedure of washing the tissue. The time consumed in making the transfers also rendered it impossible to check glycolysis at a definite period. Furthermore it was difficult to remove all the tissue from the flask. It was thought probable that, as in other tissue, the nitrogen content would be in a fairly definite relation to the dry weight and that even in case of variation due to the presence of more or less fatty tissue, it would be as accurate a measure of the active cells as would the dry weight.

As a matter of fact it was found that, with slight variations in different tumors, the nitrogen content was a very accurate measure of the amount of tissue. Table I gives the relationships between the nitrogen and the dry weight of eight different tumors. Since the amount of available tissue was small, the average figure of 10.3, obtained from the table, was used as a factor to calculate the dry weight of the tissue used in subsequent observations.

The  $\text{HgCl}_2$  precipitates were filtered and the precipitate was washed once with a small amount of dilute  $\text{H}_2\text{SO}_4$  to remove excess chloride. It was then washed into a Kjeldahl flask and digested with concentrated  $\text{H}_2\text{SO}_4$ . The mercury served as a catalyst and digestion was complete in a short time. The mercury was removed with sodium sulfite. In the early experiments the digested mixture was made to a definite volume and ammonia determinations were made on duplicate aliquots. This procedure proved unnecessary since duplicate determinations were made with two separate samples of tissue in each case. This is suggested

as an accurate method of determining small amounts of tissue. The chloride which is present does not appreciably interfere with the determination.

Owing probably to the length of time that the slices of tissue were in Ringer's solution before transfer to the reaction flasks, the amounts of lactic acid, free phosphate, and free sugar present in the tissue were negligible. To avoid the necessity, therefore, of using an extra sample of tissue, the determination of the initial amounts of lactic acid and glucose was omitted. The recorded values represent the total lactic acid at the end of the incubation period. Table II presents the initial content of several samples as prepared for an experiment. It can be seen from this that the

TABLE I.  
*Relation of Nitrogen to Dry Weight.*

Cancer.	Dry weight.	N	$\frac{\text{Dry weight}}{\text{N}}$
	<i>mg.</i>	<i>mg.</i>	
Rous.....	369	35.6	10.3
" .....	421	42.4	9.9
" .....	318	30.2	10.5
Jensen.....	110	10.5	10.5
" .....	94	9.6	9.8
Mouse.....	53	5.2	10.2
" .....	46	4.3	10.7
" .....	39	3.7	10.5
Average.....			10.3

correction is small. Since only free sugar was determined, no correction of the glucose figures can be made for the higher carbohydrate which might have been present in the tissue.<sup>1</sup>

In our early experiments we used the Rous chicken sarcoma. This contained, however, so much necrotic tissue that it was with the greatest difficulty that sufficient satisfactory material could be obtained. Further objection to the Rous tumor lay in its infiltra-

<sup>1</sup> That this had little effect is shown by the constant level which was maintained in the Warburg experiment before glucose was added, indicating little if any power of the tissue to produce lactic acid without the addition of glucose.

tion of muscle and the difficulty of separating accurately at the time of the experiment the cancer from the muscle tissue. Since the cells of both muscle and cancer have great but unequal glycolytic power, any contamination was sufficient to invalidate an experiment. In a few observations Jensen sarcoma was employed. The variability of its glycolytic activity was considerable and the frequency of spontaneous regression was a source of constant annoyance. The most satisfactory tissue was obtained from mouse sarcoma, No. 180, a strain of which was furnished us through the courtesy of Dr. J. B. Murphy of the Rockefeller Institute.

Since for chemical analysis a gm. or more of sliced tissue was often required, great difficulty was encountered in obtaining

TABLE II.  
*Mouse Sarcoma.*

Mg. per gm. dry tissue.		
Lactic acid.	Glucose.	Phosphate.
2.8	0.22	0.13
1.9	0.21	0.19
1.2	0.19	0.10
2.2	0.09	0.08
1.1	0.08	0.12
2.0	0.12	0.11

sufficient material from any single tumor and it was frequently necessary to use several tumors for one experiment. Approximately equal amounts of tumor tissue were placed in the experimental and control flasks. In order to obtain comparable results, adjoining slices of tissue were taken for experiments and control. This precaution was absolutely essential because of the irregular distribution of necrotic areas and the great variability of glycolytic activity in different tumors of the same strain.

#### EXPERIMENTAL.

For the first experiments, Rous sarcoma was employed. Through the courtesy of Dr. E. F. Du Bois they were performed with the Warburg apparatus of the Russell Sage Institute of Pathology. The results of three experiments are presented in Table III.



In the first observation, in which only 1 cc. of the pancreatic extract was used, there was no inhibition. With larger amounts of the inhibitor the glycolytic activity of the sarcoma was somewhat diminished. It will be seen that this same pancreatic

TABLE III.

*Effect of Lactic Acid Inhibitor upon Glycolysis of Rous Sarcoma.*

Date.	CO <sub>2</sub> production per mg. tissue per hr.			Remarks.
	Without inhibi- tor.	With inhibi- tor.	Per cent inhibi- tion.	
1886				
	c.mm.	c.mm.		
July 25.....	30.1	33.4	-10.0	1 cc. inhibitor.
" 27.....	28.4	24.1	+15.1	1.5 " "
" 27.....	22.0	16.5	+25.0	3.0 " "

This inhibitor produced by chemical analysis 50.3 per cent inhibition upon glycolysis of muscle tissue.

TABLE IV.

*Effect of Lactic Acid Inhibitor upon Glycolysis of Jensen Rat Sarcoma.*

Date.	CO <sub>2</sub> production per mg. tissue per hr.			Per cent inhibition on muscle tissue.
	Without inhibitor.	With inhibitor.	Per cent inhibition.	
1927	c.mm.	c.mm.		
Mar. 15.....	31.9	30.9	3.2	56.0
“ 22.....	33.5	20.9	37.6	
“ 25.....	39.3	33.4	16.0	48.2
“ 29.....	44.0	41.2	6.3	56.0
Apr. 8.....	22.6	24.9	-10.0*	48.0
“ 20.....	27.4	27.3	0.0	

\* Inhibition of glycolysis in Jensen sarcoma was 17.0 per cent by chemical analysis.

preparation was sufficiently active to produce an inhibition of 50.3 per cent upon the glycolysis of muscle tissue.

In our own laboratory the experiments were repeated on Jensen rat sarcoma. These are shown in Table IV. While the results are somewhat more variable, the degree of inhibition was considerable only in one experiment. In the other observation the glycolytic

activity was checked to an almost negligible degree by extracts which had been shown to have a highly inhibitory effect on the glycolysis of muscle tissue. From these experiments it was evident that the inhibition of glycolysis in cancer tissue was by no means as complete as in muscle. The results were, however, not sufficiently decisive to permit definite conclusions. Furthermore, a considerable source of error was present in these first observations because no determination was made of the retention factor in the

TABLE V.  
*Effect of Injury and Extraction on Glycolytic Activity of Rous Sarcoma.*

Condition.	Lactic acid, mg. per gm. dry tissue.			
	Sliced tissue.	Finely hashed tissue.	Thoroughly ground tissue.	Extract.
Before incubation.....		19.70	31.30	2.84
After ".....		23.50	31.60	2.81
Before incubation.....	20.2		43.6	18.8
After ".....	110.3		42.9	18.3

TABLE VI.  
*Glycolysis of Mouse Sarcoma 180 with Glucose and Glycogen.*

Date.	CO <sub>2</sub> production per mg. tissue per hr.	
	With glucose.	With glycogen.
<i>1928</i>	<i>c.mm.</i>	<i>c.mm.</i>
Mar. 20.....	15.7	0.3
Apr. 19.....	12.0	1.0
May 8.....	10.5	0.9

reaction mixtures to which the inhibitor was added. In later experiments, the retention factor was variable, ranging between 1.00 and 1.17. If it had been determined, the measured CO<sub>2</sub> production would have been somewhat greater and the apparent inhibition would have been less.

A few months after the first of our experiments was performed Meyerhof (4) announced the discovery of a muscle extract which could be prepared by crushing chilled muscle and extracting it with water. It was found, moreover, that this extract could not use

glucose except under special conditions and was most active when glycogen was added to the reaction mixture.

Similar treatment of cancer tissue was attempted. In Table V experiments on Rous sarcoma will be found. Cancer cells were ground in a mortar with a small amount of water to plasmolyze them and enough glucose was added to give a concentration of 0.2 per cent. No attempt was made to chill the tissue since Warburg (11) in his original experiments had shown that freezing completely destroys the glycolytic activity of cancer. To prepare the extract, ground tissue was diluted with 10 cc. of water for each 5 gm. of cancer. 3 cc. of this were diluted with 1 cc. of water containing glucose. The extract thus obtained was filtered through muslin. 15 cc. of water were added to the filtered extract which was then made up to a total of 20 cc. with bicarbonate and glucose sufficient to give to the whole solution a bicarbonate concentration of 0.025 M and a glucose concentration of 0.2 per cent. In Table V it will be seen that thorough grinding and extraction with water destroys completely the glycolytic power of cancer tissue. On the other hand, hashing, which does not destroy all of the cells, inhibits greatly but does not entirely prevent glycolysis.

It was found that cancer tissue under the experimental conditions is incapable of using glycogen as is shown in the experiments of Table VI. The activity of the extracts is indicated by the degree of glycolysis which occurs when glucose is present. It will be seen that with glycogen the  $\text{CO}_2$  formation is negligible and possibly within the limits of experimental error. This may be due to impermeability of the intact cells to glycogen and not to an inability to use glycogen once it is within the cell.

Some observations were made on the relation between the disappearance of glucose and the production of lactic acid. The first experiments were performed with Rous sarcoma and were later repeated with Mouse Sarcoma 180. The original results are shown in Table VII. A comparison was made between the activity of cancer tissue in oxygen, in nitrogen, and in air with 0.001 M KCN added to the reaction mixture. As in Warburg's experiments the lactic acid production in nitrogen was found to be great and was equal to that which occurred when the tissue was exposed to KCN. In oxygen the lactic acid formation was considerable but much less than under the other conditions. In all

TABLE VII.  
*Glycolysis in Rous Sarcoma. Comparison of Lactic Acid and Glucose Changes.*

Condition.		Lactic acid increase. mg.*	Glucose lost. mg.*	Ratio glucose lactic acid
Reaction mixture.	Gas mixture.			
Experiment I.				
Sample 1. Ringer's bicarbonate with 0.25 per cent glucose.	N <sub>2</sub> + 5 per cent CO <sub>2</sub> .	79.4	108.0	1.36
" 2. Same with KCN.....	Air + 5 " "	73.3	112.1	1.53
" 3. " as Sample 1.....	O <sub>2</sub> + 5 " "	49.7	130.3	2.71
" 4. Ringer's bicarbonate with 0.25 per cent glucose.	N <sub>2</sub> + 5 " "	78.8	110.4	1.42
" 5. Same with KCN.....	Air + 5 " "	80.5	113.3	1.40
Experiment II.				
Sample 1. Ringer's bicarbonate with 0.25 per cent glucose.	N <sub>2</sub> + 5 per cent CO <sub>2</sub> .	67.0	109.3	1.62
" 2. Same with KCN.....	Air + 5 " "	68.9	111.9	1.62
" 3. " as Sample 1.....	O <sub>2</sub> + 5 " "	58.0	121.6	2.10
Experiment III.				
Sample 1. Ringer's bicarbonate with 0.25 per cent glucose.	N <sub>2</sub> + 5 per cent CO <sub>2</sub> .	103.8	189.5	1.82
" 2. Same with KCN.....	Air + 5 " "	111.4	183.6	1.61
" 3. " as Sample 1.....	O <sub>2</sub> + 5 " "	83.1	206.3	2.48
" 4. Ringer's bicarbonate with phosphate buffer and 0.2 per cent glucose.....	N <sub>2</sub> + 5 " "	106.7	180.4	1.73

\*Per gm. dry tissue.

of our experiments, the glucose loss was greater than the lactic acid production. The ratios are shown in the last column of Table VII.

It is surprising that while in an atmosphere of oxygen the production of lactic acid is much diminished, the loss in glucose is considerably greater than in nitrogen or KCN. Although it seems possible that this greater loss of glucose may be attributed to oxidation of carbohydrate, differential relationships are quite impossible to determine because of the complicated character of the reactions. Glucose is being broken down by the glycolytic enzymes of the cancer. Some glucose is presumably being oxidized. According to the present theories, one may suspect that in the presence of oxygen some of the performed lactic acid is being reconverted into its precursor. Oxidative processes, however, cannot explain the discrepancy between destruction of carbohydrate and formation of lactic acid in an atmosphere of nitrogen or after the addition of potassium cyanide. In the light of experiments on muscle and muscle extract, it might be assumed that the extra loss of carbohydrate was due to the formation of hexosephosphate or some carbohydrate-phosphate combination. The phosphate content of tumors, however, is very low, extraction of 0.5 to 1.0 gm. of tissue with trichloroacetic acid yielding less than 0.05 mg. of phosphate. As will be shown in later experiments the phosphate changes during glycolysis are so extremely small as to be scarcely measurable even when large amounts of phosphate are added to the reaction mixture. In Table VII, Experiment III shows that the addition of phosphate to the Ringer bicarbonate glucose solution caused no change in the glycolytic activity of the tissue either in the glucose loss or lactic acid production.

Ronzoni, Glaser, and Barr (6) find that in muscle the pancreatic inhibitor acts only on one phase of glycolysis. It prevents completely the formation of a carbohydrate phosphate ester, probably hexosephosphate, but has no effect whatever upon breakdown of this substance when preformed.

The experiments on cancer show that the differences between the glycolysis of malignant tumors and of muscle are extremely great. Muscle extract prepared by crushing chilled muscle tissue has only slightly less activity than the muscle itself. Injury to the muscle

increases the rate of lactic acid formation as does stimulation. The extract breaks down glycogen but has no effect on glucose. On the other hand, hashing of cancer tissue diminishes its activity. Freezing, grinding, and extracting destroy all of its glycolytic power. The tumor cells destroy glucose but have no effect on added glycogen. Furthermore, it has been shown that the forma-

TABLE VIII.

*Carbohydrate, Lactic Acid, and Phosphate Changes in Mouse Sarcoma with and without Pancreatic Inhibitor.*

Condition.	Loss of carbohy- drate per gm. dry weight.	Lactic acid gain per gm. dry weight.	Phosphate change per gm. dry weight.	Remarks.
	mg.	mg.	mg.	
With inhibition.	63.2	35.1	+0.79	Mixed samples of same num- ber of slices from each of three tumors slightly nec- rotic. Incubated 3 hrs. at 37.5° with phosphate buffer.
	61.3	32.6	+0.65	
Without inhibition.	58.9	28.3	+0.29	
	62.6	34.3	+0.30	
Three tumors; phosphate buffer.				
With inhibition.	106	57.0	+0.63	Samples from three tumors as above. Incubated 3 hrs. at 37.5° in phosphate buffer.
	112	62.0	+0.51	
Without inhibition.	103	74.0	+0.32	
	108	68.0	+0.38	
Tumor I.	284	162.0	+0.36	Excellent tissue, both samples from same tumor. Incu- bated 3 hrs. at 37.5°.
	256	151.6	+0.41	
Tumor II.	390.2	212.1	+0.32	Excellent tissue, both sam- ples from same tumor. In- cubated 3 hrs. at 37.5°.
	370.6	215.0	+0.49	

tion of lactic acid in muscle is accomplished through an intermediate stage involving a carbohydrate-phosphate combination. In cancer, no evidence of such an intermediary has been obtained. With such striking differences in glycolysis, it became increasingly unlikely that the pancreatic factor so potent in muscle could have any specific effect on the glycolysis of a tissue in which no evidence of hexosephosphate formation had been discovered.

Our original experiments, however, were indecisive. We felt, moreover, that the whole question of glycolysis is much too complicated to allow certain predictions from theoretical considerations. Further experiments, both chemical and gasometric, were therefore performed. The chemical results are shown in Table VIII. It will be seen that in these experiments there is again

TABLE IX.  
*Glycolysis of Tumor Tissue with and without Inhibitor.*

Date.	Tumor.	Manometric method (Warburg). CO <sub>2</sub> per mg. per hr.		Chemical methods. Mg. per gm. dry tissue.			
		Without inhibitor.	With inhibitor.	Without inhibitor.		With inhibitor.	
				Lactic acid.	Glucose.	Lactic acid.	Glucose.
1928		c.mm.	c.mm.				
Jan. 12	Mouse Sarcoma 180.	17.4 15.5	26.1 19.5	113.0 122.0	145.0 152.0	124.5 130.0	160.0 168.0
Jan. 18	Mouse Sarcoma 180.	3.8* 3.6*	7.9* 4.6*	164.2 100.4	187.0 118.0	164.4	183.0
Feb. 24	Jensen sarcoma.	17.7 22.9 22.5	17.7 20.2 16.0	140.0 110.0	144.0 123.0	146.0 127.0	152.0 126.0
Mar. 2	Mouse Sarcoma 180.	19.8 21.2	23.2 26.5	305.0 213.0	448.0 324.0	289.0 151.0†	354.0 164.0†
Mar. 20	Mouse Sarcoma 180.	15.7 16.1	17.8 16.2				

\* The tumor tissue used in the Warburg apparatus was necrotic.

† Weight of tissue probably wrong. Much greater than other although the weights of duplicate samples usually checked very closely.

evidence of a greater destruction of carbohydrate than can be accounted for by the production of lactic acid. One may also see the extremely small changes which take place in the content of free phosphates even when phosphate buffer is added. Even under the influence of NaF there are no demonstrable phosphate changes. Finally, it will be observed that the inhibition either of carbohydrate or lactic acid changes is negligible.

In Table IX there are presented results obtained both with the manometric method of Warburg and by chemical methods. Although chemical and gasometric determinations were made simultaneously, a comparison of the lactic acid formation indicated by the two methods is quite impossible under the conditions of our experiments. Different parts of the same tumor and often different tumors were used in the two sets of experiments. The variability of glycolytic power in the different tumors was great. The difficulty occasioned by patches of irregular necrosis was never entirely overcome. Another complication arises in making any comparison. It has been shown that the glucose destruction always exceeds the lactic acid formation and no adequate explanation of the discrepancy has been offered. From this extra glucose it is entirely possible that substances are formed which may influence the rate of carbon dioxide production. This or similar factors may explain the greater variability of the results obtained by the manometric method.

In only one of the experiments of Table IX was there any considerable inhibition either of glucose or lactic acid change. The accumulated evidence seems to be overwhelming that the inhibitor has no specific or constant effect on the glycolysis of cancer tissue.

#### CONCLUSIONS.

1. Study has been made of the action of the pancreatic inhibitor on the glycolysis of cancer tissue, both by the manometric method of Warburg and by chemical determination of glucose, lactic acid, and phosphate changes.
2. The inhibitor which is so active in preventing the glycolysis of muscle and muscle extract has no effect on cancer tissue.
3. The nature of glycolysis in malignant tumors differs in many important respects from that of muscle. Hashing diminishes the glycolytic activity of cancer tissue. Grinding, freezing, or extraction destroys it completely. Glycogen cannot be utilized in the glycolysis of cancer cells. The phosphate content of tumors is low and there is no evidence that hexosephosphate or other combinations of carbohydrate and phosphate are formed in cancer.



4. Since the pancreatic inhibitor exerts its effect in muscle by preventing the esterification of carbohydrate and phosphate, its failure to affect the glycolysis of cancer tissue is satisfactorily explained.

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# PREPARATION OF SOME SUBSTITUTED GUANIDINES.

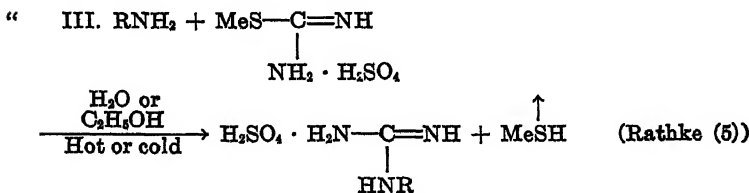
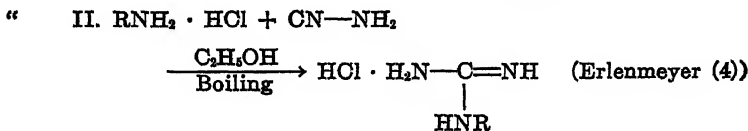
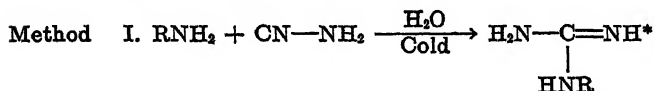
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## INTRODUCTION.

In studying the nature of the hypoglycemic action of guanidine compounds, it was found necessary to prepare a number of guanidine derivatives not obtainable and in many instances not described in the literature. This paper concerns itself with syntheses in which the original material was an amine or imine. The preparation of diguanylpiperazine, guanylpiperidine, guanyl-*p*-aminodimethylaniline, diguanidinopentamethylene, and diguanidinooctamethylene is recorded. No new principle has been developed, the general methods of guanidine preparation having been adapted. These may be summarized as follows:

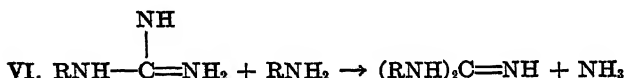
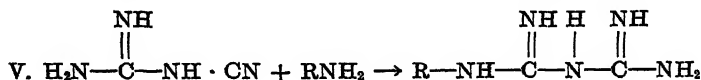
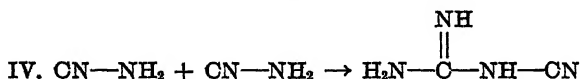



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\* We do not know whose name is associated with Method I. It was used in converting amino acids to guanidino acids by Engeland and Kutscher (1). Ripke (2) and Kiesel (3) prepared diguanidinopentamethylene and agmatine in this manner.

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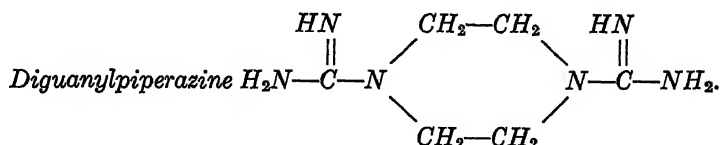
Side reactions are possible in each instance. The three most common side reactions and the ones encountered in these preparations are the polymerization of cyanamide to dicyanodiamide, the condensation of this latter compound with the parent amine to give a diguanide, and the reaction of the desired mono-substituted guanidine with unchanged parent amino base to give a di-substituted product. These may be outlined as follows:



Side reaction (IV) takes place readily in alkaline solution and is consequently met with in Method I. It would appear that Method I becomes practical when the rate of condensation for the guanidine compound exceeds the dicyanodiamide polymerization. According to Werner and Bell (6) dicyanodiamide acts potentially as cyanamide, methylguanidine being obtained when dicyanodiamide is heated with methylamine hydrochloride at 180°. No instance of this reaction was encountered by us at room temperature or 100°. The conditions under which reaction (V) takes place were observed by Cohn (7) and Bamberger and Seeberger (8). The mechanism of reaction (VI) is not clearly understood and is apparently not as simple as represented by equation (VI). In some instances this reaction may be induced to take place almost quantitatively (9). It has been studied at elevated temperatures by Klinger (10). An excellent discussion is given by Schotte *et al.* (11).

A survey of the recent guanidine literature indicates the popularity of methylisothiurea sulfate, Method III, over the older cyanamide condensations, Methods I and II. In our experience, the methylisothiurea sulfate method is practical when the desired guanidine sulfate is sufficiently insoluble as to crystallize out from

the reaction mixture. It is impractical when an alkaline silver separation is required for purification.



Four modifications of conditions were tried out in attempting to prepare diguanylpiperazine. When piperazine hydrochloride was refluxed in absolute alcohol with cyanamide, the hydrochloride of diguanylpiperazine was isolated (Method II). Piperazine hydrate was found to react with methylisothiurea sulfate in aqueous solution at the boiling point of water and even at room temperature, but the isolation of the pure sulfate was unsuccessful. In crystallizing from water, the salt was contaminated with methylisothiurea sulfate. An alkaline silver separation, with subsequent conversion to the hydrochloride and chloroplatinate, yielded a precipitate which analyzed too high for platinum and melted  $40^\circ$  below the chloroplatinate obtained by Method I described above. When piperazine hydrate was permitted to react with cyanamide in aqueous solution at room temperature, 40 per cent of the cyanamide crystallized out as dicyanodiamide in 10 days standing (m. p.  $204\text{--}206^\circ$ ). When the solution was concentrated, more dicyanodiamide crystallized out, and when alcohol was added, unchanged piperazine hydrate was precipitated.

*Preparation.*—15.0 gm. of piperazine hydrochloride, made by evaporation of the Eastman hydrate with hydrochloric acid and 11.0 gm. of cyanamide, were refluxed in 250 cc. of absolute alcohol for 24 hours. After the mixture was cooled, the insoluble residue was filtered off, 9.5 gm. This analyzed 38.9 per cent HCl. The alcoholic filtrate was evaporated to dryness, and the semi-crystalline mass obtained, crystallized from water, 6.5 gm. The crystals analyzed 24.1 per cent HCl. Both of these products were recrystallized from 20 cc. of hot water. In the case of the alcohol-insoluble residue, solution was complete, while in the alcohol-soluble fraction an amorphous precipitate was filtered off. The filtrate of this product yielded large crystalline clusters, 2.7 gm., and a fine amorphous precipitate upon cooling. The amorphous

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precipitate was decanted off. The crystals (5.5 gm.) obtained from both the hot water solutions, crumpled to a white powder on drying, and did not blacken or melt up to 310°.

*Analysis.*— $(\text{CN}_2\text{H}_3)_2(\text{CH}_2)_4\text{HCl}$ .

Calculated. HCl 30.0.

Found. " (Volhard) 30.3, 30.1.

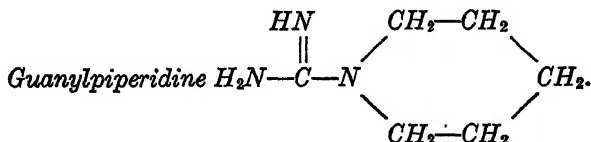
The two products were converted to the chloroplatinates by precipitation with  $\text{PtCl}_4$  from aqueous solution. The very insoluble precipitates were washed several times with water and dried at 110°. There were blackening and frothing at 279–280°.

*Analysis.*— $(\text{CN}_2\text{H}_3)_2(\text{CH}_2)_4\text{H}_2\text{PtCl}_6$ .

Calculated. N 14.5, Pt 33.6.

Found. " (Dumas) 14.6, " 33.7.

The yield of diguanylpiperazine hydrochloride was only 22 per cent of theory.



Guanylpiperidine was readily prepared in aqueous solution at room temperature from piperidine and methylisothiourea sulfate (Method III). By concentration of the reaction mixture, pure crystals of the desired compound crystallized out. The reaction may be accelerated by raising the temperature. The reaction of cyanamide with piperidine in aqueous solution, with subsequent removal of the excess piperidine by steam distillation, yielded a resinous product from which the pure guanylpiperidine sulfate was not isolated. It is highly probable that



much piperylbiquanide,  $\text{NH}_2-\text{C}-\text{NH}-\text{C}-\text{NC}_5\text{H}_{10}$ , is formed under these conditions. Bamberger and Seeberger (8) prepared piperylbiquanide by heating dicyanodiamide with piperidine in aqueous solution. They showed further that the reaction takes place at room temperature. Since cyanamide condenses to dicy-

anodiamide in alkaline solution, the conditions of our condensation were similar to those of Bamberger. We were unable to isolate pure piperylbisguanide, but two constants of crystals, obtained by fractional crystallization from alcohol, indicated its presence; *viz.*, a sulfate which melted below  $180^{\circ}$  and analyzed 31.6 per cent  $\text{H}_2\text{SO}_4$ .

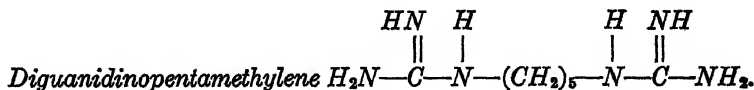
*Preparation.*—Eastman crude piperidine was used in the trial condensation. Kahlbaum's highly purified piperidine was then substituted in duplicating the successful synthesis. Because of the unusual physiological properties revealed by the first sample of guanylpiperidine obtained, it was considered expedient to prepare the compound from another starting material, in order to minimize the possibility that traces of an impurity carried the physiologically active group.

5 gm. of piperidine and 13 gm. of methylisothiurea sulfate were dissolved in 50 cc. of water. After standing 3 days, the volume of the solution was taken down to 30 cc. when crystals formed, 3.7 gm. The reaction of the solution was alkaline. In several trials dilute sulfuric acid was added at this point, with the result that the crystals which formed were contaminated with methylisothiurea sulfate, and recrystallization from water was necessary for purification. In the cases where the reaction mixture was left alkaline, recrystallization was unnecessary. It is to be expected that in the distribution of the sulfuric acid between the guanylpiperidine and the unchanged methylisothiurea, the stronger base should take up the preponderance of available acid. The crystals which formed were filtered off and washed with ice water. By concentration of the mother liquor to 20 cc., another crop of crystals formed. Yield 40 per cent.

*Analysis.*— $(\text{CN}_2\text{H}_3\text{C}_2\text{H}_5)_2\text{H}_2\text{SO}_4$ .

Calculated.	N	23.9,	$\text{H}_2\text{SO}_4$	27.9.
Found.	" (Dumas)	24.2,	"	27.9.

The guanylpiperidine sulfate did not melt below  $270^{\circ}$ , where it commenced to char. The picrate is very insoluble, forming in a dilution of 1:2000. It decomposes at  $242\text{--}243^{\circ}$ .



Ripke (2) obtained this compound by the reaction of cyanamide with pentamethylenediamine in aqueous solution at room temperature for 17 days. The impure sulfate was isolated from the reaction mixture by the conventional alkaline silver salt separation. By conversion of the sulfate to the chloride, a gold salt which gave the correct analytical values was obtained.

We have applied the Erlenmeyer synthesis (Method II (4)) to the preparation of the compound. The whole procedure may be carried out in 2 days with an increased yield over the Ripke method and the elimination of the tedious alkaline silver salt separation. As did Ripke, we obtained a sulfate which analyzed high for sulfur, but which gave excellent values when converted to the chloroaurate.

*Preparation.*—Pentamethylenediamine hydrochloride was prepared by the method of Ladenburg (12) with Eastman dicyanotrimethylene. 10.0 gm. of pentamethylenediamine hydrochloride, 9.0 gm. of cyanamide, and 150 cc. of absolute alcohol were refluxed for 12 hours. The alcohol was then evaporated off, an oily syrup remaining. This was dissolved in the minimum amount of water, 6.0 gm.,  $\text{Na}_2\text{CO}_3$  added, and the solution evaporated on the water bath to dryness. The residue was extracted with absolute alcohol, the insoluble material being discarded. A cold mixture of concentrated sulfuric acid and absolute alcohol was then added until no more precipitate formed. The crystals were filtered off, recrystallized from water, and dried at  $105^\circ$ . Yield 8.0 gm. (47 per cent).

*Analysis.*— $(\text{CN}_2\text{H}_4)_2(\text{CH}_2)_5\text{H}_2\text{SO}_4$ .

Calculated.	N	29.6,	$\text{H}_2\text{SO}_4$	34.5.
Found.	" (Dumas)	27.0,	" by $\text{BaSO}_4$	35.4.

By concentration of the mother liquor, an additional crop of crystals was obtained. These upon analysis gave 27.8 per cent N and 37.1 per cent  $\text{H}_2\text{SO}_4$ . The impure sulfate decomposed at  $285^\circ$ . The chloroaurate was prepared by dissolving 1 part of the sulfate in 8 parts of water and adding 105 per cent of the theoretical amount of  $\text{BaCl}_2$  to precipitate the  $\text{H}_2\text{SO}_4$ . The  $\text{BaSO}_4$  was centrifuged off and  $\text{AuCl}_3$  added to the filtrate. The double gold salt precipitated immediately as microscopic crystals. It was washed with water and dried at  $100^\circ$ .

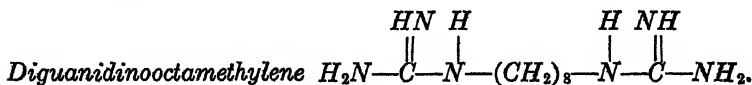
*Analysis.*— $(\text{CN}_2\text{H}_4)_2(\text{CH}_2)_8(\text{HAuCl}_4)_2$ .

Calculated. N 9.7, Au 45.6

Found. " (Dumas) 9.5, " 45.7.

The diguanidinopentamethylene chloraurate shrinks at  $139.5^\circ$ , melting at  $151^\circ$ .

Ripke's synthesis and alkaline silver separation were duplicated with the same pentamethylenediamine used in the method described above. 6 gm. of sulfate were obtained from 10.0 gm. of pentamethylenediamine hydrochloride, equivalent to a yield of 35 per cent. The melting point of the chloraurate obtained by this procedure was  $147^\circ$ . One crystallization from alcohol-water raised the melting point to  $151^\circ$ , the same value obtained by our method. Ripke gives the melting point of the chloraurate as  $161^\circ$ . We were unable to raise the melting point of our chloraurates by three crystallizations. Since our analytical values are in closer agreement with the theoretical values than those obtained by Ripke, we are at a loss to explain this difference in melting points. It should be noted, however, that the lowering of the melting point of the chloraurates of the guanidine bases by impurities is more marked than it is for the chloroplatinate or the picrates. We found this to be true in the isolation of the guanidine base synthalin in the commercial preparation, glukhorment (13). In this instance, the first chloraurate melted  $8^\circ$  below the melting point for the pure compound, the chloroplatinate and picrate only  $2^\circ$  lower.

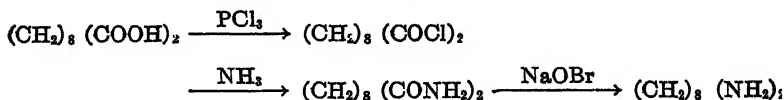


This compound has probably been prepared by the synthalin workers but no details have appeared in the literature. We used diaminooctamethylene hydrochloride as the initial material. An attempt to convert this compound to the diguanidino compound by refluxing in absolute alcohol with cyanamide was unsuccessful, the cyanamide going over to dicyanodiamide (recovery of 80 per cent of the cyanamide added as dicyanodiamide, m. p.  $205^\circ$ ). The desired compound was readily prepared in aqueous solution and at room temperature from the free amine base and methylisothiurea sulfate.



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*Preparation.*—Diaminooctamethylene was prepared from Eastman sebacic acid, by the following scheme, outlined in Beilstein.



The diaminooctamethylene was separated from the hypobromite reaction mixture by steam distillation and converted to the hydrochloride. This formed a platinum salt which upon analysis gave 35.2 per cent Pt (theory 35.3 per cent Pt). 3.1 gm. of diaminooctamethylene hydrochloride and 1.0 gm. of caustic were dissolved in 11 cc. of water. To this solution were added 6.0 gm. of methylisothiourea sulfate in 42 cc. of water. After it had stood at room temperature for 7 days, 1.4 gm. of crystals were filtered off. After concentration of the solution, another 1.0 gm. of crystals was obtained. The combined crystals were dried at 110°. Yield 60 per cent.

*Analysis.*— $(\text{N}_2\text{CH}_4)_2(\text{CH}_2)_8\text{H}_2\text{SO}_4$ .

Calculated.	N	25.0, $\text{H}_2\text{SO}_4$	30.0.
Found.	" (Dumas)	25.0, " by $\text{BaSO}_4$	29.6.

The sulfate was converted to the chloride by the addition of the theoretical amount of  $\text{BaCl}_2$ . The platinum salt was precipitated from the chloride in aqueous solution. Upon analysis this gave 30.5 per cent Pt (theory 30.6 per cent Pt), m. p. 214–216°. The picrate was precipitated from an aqueous solution of the sulfate m. p. 205–206.5°. The gold salt was thrown down in aqueous solution, m. p. 143–144°. Fractional crystallization of the gold salt from water gave products with identical melting points.

### *p*-Guanidinodimethylaniline.

The condensation of *p*-aminodimethylaniline with cyanamide takes place readily, either in boiling alcohol as the hydrochloride or in water as the free base. The reaction product is contaminated with highly colored impurities. These may be separated by fractional crystallization of the sulfate from water. The base is very soluble in water, alcohol, or ether. The hydrochloride nitrate, and sulfate are very water-soluble.

*Preparation.*

*Condensation in Alcohol.*—17 gm. of *p*-amino-dimethylaniline hydrochloride, 300 cc. of alcohol, and 4.5 gm. of cyanamide were refluxed 5 hours. A clear solution resulted. The alcohol was then distilled off, 50 cc. of water added, and concentrated NaOH to make strongly alkaline. The solution was then extracted three times with a total volume of 500 cc. of ether. The ether was evaporated off under a vacuum, leaving a dark brown viscous oil which crystallized within several days. The crystals were separated from the oil by being washed by decantation with a little water and alcohol. M. p. 117–122°.

*Analysis.*— $(\text{CH}_3)_2\text{NC}_6\text{H}_4\text{N}_2\text{CH}_4 \cdot \text{H}_2\text{O}$ .

Calculated. N 28.6.

Found. “ (Kjeldahl) 28.6, 27.9.

Larger yields of the purified compound are obtained by converting the crude ether extract to the nitrate or sulfate and crystallizing fractionally from water. The nitrate was obtained as prisms, m. p. 185°. The sulfate crystallizes in large rhombohedral aggregates, m. p. 112–112.5°.

*Analysis.*— $(\text{CH}_3)_2\text{NC}_6\text{H}_4\text{N}_2\text{CH}_4\text{H}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$ .

Calculated. N 17.9,  $\text{H}_2\text{SO}_4$  31.4.

Found. “ (Kjeldahl) 17.7, 17.3, “ by  $\text{BaSO}_4$  31.3, 31.1.

The chloroplatinate was obtained from the crude ether extract by fractional precipitation in water-alcohol. Upon addition of  $\text{PtCl}_4$  to a 70 per cent solution of the crude hydrochloride, purple crystals precipitated immediately. These gave upon analysis 34.2 per cent Pt and 7.0 per cent N. When the filtrate was evaporated to two-thirds the original volume, yellow crystals containing the required amount of Pt, 33.2 per cent, were obtained. The purple chloroplatinate, which was isolated several times from different water-alcohol concentrations, always had the same Pt content; viz., 34.2 per cent Pt. This approximates the theoretical value for a compound containing 1 cyanamide molecule for 2 dimethylaminoaniline molecules.

*Condensation in Water.*—17 gm. of hydrochloride, 5.2 gm. of  $\text{Na}_2\text{CO}_3$ , 5.0 gm. of cyanamide, and 140 cc. of water were autoclaved 5 hours at 110°. The purification was the same as for the alcohol condensation.

*Diguanylbenzidine.*

Diguanylbenzidine is not described in the literature, though the isomeric biguanide compound and the dibiguanide have been prepared (7). We were unable to isolate diguanylbenzidine in a pure state by refluxing benzidine hydrochloride and cyanamide in absolute alcohol. Much unchanged benzidine was always recovered even though an excess of cyanamide was used. The fraction which reacted with the cyanamide was relatively cold water-soluble, and by conversion to the sulfates, a cold water-soluble sulfate and hot water-soluble sulfate fractionation was made. The nitrogen values for these fractions indicated that the cold water-soluble sulfate consisted mainly of diguanylbenzidine sulfate and the hot water-soluble sulfate of monoguanylbenzidine sulfate. Fractional crystallization of these products showed, however, that they contained more than one compound. Attempts to isolate the pure derivatives were unsuccessful.

A summary of the constants of some of these basic fractions is included. The calculated nitrogen content of monoguanylbenzidine is 24.9 per cent N; and for diguanylbenzidine 31.4 per cent N.

Fraction A, relatively water-insoluble, dilute HCl-soluble, alcohol-insoluble, 23.6 per cent N. Not melting below 230°. Fraction B, relatively water-insoluble, dilute HCl-soluble, alcohol-soluble, 24.5 per cent N. Melting with decomposition at 205°. Chloroplatinate analyzed 27.5 per cent. Fraction C, dilute HCl-soluble, dilute H<sub>2</sub>SO<sub>4</sub>-soluble, hot, 26.6 per cent N. Charring at 215°. Does not melt up to 240°. Fraction D, relatively water-, and dilute H<sub>2</sub>SO<sub>4</sub>-soluble, cold. M. p. 187°. 28 to 32 per cent N. Sulfate analyzed 18 per cent N.

It should be noted that certain of these fractions had a physiological action similar to synthalin. Benzidine was found to be unreactive with methylisothiourea sulfate in aqueous alcohol solution in the cold.

*Negatively Substituted Aromatic Amines.*

Kampf (14) makes the statement that cyanamide will not react with aromatic amines in alcohol solution, it being necessary to use salts of the bases. He further points out that negatively substituted amines will not react under either condition. We confirmed his observation in the case of *p*-nitroaniline, pure un-

changed *p*-nitroaniline HCl being recovered from an absolute alcohol solution which had been refluxed for several days with cyanamide. The effect of the negative group was found to extend even to a compound such as *p*-aminoacetanilide. The hydrochloride of this base remained unchanged when refluxed with cyanamide in alcohol solution, and the free base unchanged when boiled with cyanamide in water or when permitted to stand as long as a month in aqueous solution with methylisothiurea sulfate or when heated with this compound. (The unchanged base was identified by the melting point, 162°, and the platinum analysis of the chloroplatinate.)

We are indebted to Miss Elsie Hill for some of the analyses.

#### SUMMARY.

The preparation of diguanylpiperazine, guanylpiperidine, guanylp-aminodimethylaniline, and diguanidinooctamethylene is described. A new method for preparing diguanidinopentamethylene is included.

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## SEASONAL VARIATIONS IN THE IODINE AND THYROXINE CONTENT OF THE THYROID GLAND.

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One of the most significant problems related to the chemistry of the thyroid gland is whether or not more than one iodine compound is contained in the gland. The first work in this field was the initial investigation of Baumann (1) in 1896, during which he discovered that iodine is a normal constituent of the gland. He found that acid hydrolysis separated a fraction which contained iodine and possessed the typical activity of desiccated thyroid. It amounted to less than 1 per cent of the total weight of the material, and it did not contain more than a few per cent of the total iodine contained in the desiccated gland. This fraction, iodothyron, was investigated in detail by Roos (24), Nürnberg (17), von Cyon (6), Oswald (19), and others. The highest iodine content of any sample of iodothyron which has been reported was 14 per cent, and although the yield of the total iodine which can be separated in the form of iodothyron has not been stated by many investigators, we have not been able to obtain more than 25 per cent of the total iodine in the iodothyron fraction.

The next contribution to the nature of the iodine-containing compounds was made in 1914. Kendall (14) showed that after normal thyroid material had been hydrolyzed with sodium hydroxide, approximately 50 per cent of the iodine was insoluble in dilute acid. During the early part of this investigation, although the amount of thyroxine, which was isolated was a small percentage of the total iodine, it was assumed that the failure to isolate all of the iodine in the acid-insoluble fraction as thyroxine was due to the method employed. It was also assumed that the thyroxine content of the acid-insoluble fraction was directly proportional to the iodine content.

The first suggestion that all of the iodine in the acid-insoluble compounds was not thyroxine arose from Plummer's observation (20) that from 3 to 4 times as much of this material were required to bring about the same increase in basal metabolism which was produced by pure crystalline thyroxine when the two preparations were administered according to their iodine content. At first the failure of the acid-insoluble products to produce as much effect as pure thyroxine was believed to be due to incomplete absorption of the material from the alimentary tract. Further investigation of the chemical nature of this fraction of the iodine-containing compounds suggests, however, that the physiological effect of the material is quantitatively less because not more than from 20 to 30 per cent of the total iodine is in the form of thyroxine.

In addition to the iodine-containing compounds insoluble in acid which are not thyroxine, approximately a half of the total iodine-containing compounds is soluble in acid. Ingvaldsen and Cameron (13) reported evidence which suggests that diiodotyrosine is present in the gland, although they did not isolate this substance in crystalline form. These results prove that all of the iodine cannot be present in the same form of combination and it seemed desirable to determine variations in the percentage distribution of the various iodine compounds so far as this is revealed by fractionation of the products of hydrolysis.

In 1915 Kendall described a method for the decomposition of the proteins of the thyroid gland with sodium hydroxide followed by further hydrolysis and fractionation with barium hydroxide. In 1926 Harington (9) modified the method: the hydrolysis was carried out with barium hydroxide only. By this method he was able to isolate about 14 per cent of the total iodine of a sample of desiccated thyroid in the form of thyroxine. This was higher than the percentage of the total iodine which had been separated in the form of thyroxine by Kendall. Harington assumed that some of the thyroxine was decomposed with sodium hydroxide. For many reasons this explanation did not seem satisfactory and it was decided to determine the stability of thyroxine and its derivatives in the presence of sodium hydroxide and barium hydroxide. 100 mg. of thyroxine were heated in 5 per cent sodium hydroxide solution for 24 hours. This did not break off any iodine and almost 100 per cent of the thyroxine was recovered. If the

acetyl of thyroxine is heated at 100° in 5 per cent sodium hydroxide solution, it is only slowly hydrolyzed. At the end of 18 hours 90 per cent of the thyroxine which was previously present as the acetyl was isolated as pure crystalline thyroxine. When 40 per cent barium hydroxide was used in place of sodium hydroxide, it was impossible to hydrolyze any of the acetyl; it was almost quantitatively recovered in crystalline form.

The stability of the acetyl in the presence of barium hydroxide is probably explained by the insolubility of the barium salt in the presence of 40 per cent barium hydroxide. The results of the investigation with sodium hydroxide and barium hydroxide prove that thyroxine is not destroyed by either of these alkalies. Experience with both of the alkalies has shown certain advantages and certain disadvantages which may be summarized as follows: With the material which has been available in this laboratory the solutions obtained by the treatment of fresh or desiccated material with barium hydroxide are difficult to filter, and the concentration of barium hydroxide suggested by Harington requires relatively large volumes when 45 kilos of thyroid glands are used. Furthermore, the iodine compounds are divided into two fractions, one soluble in barium hydroxide, the other insoluble. This results in either the loss of the barium-insoluble fraction or retreatment of the precipitate. We have found it a difficult matter to carry out this step.

One significant advantage is possessed by barium hydroxide; the fatty acids and dark impurities are insoluble and the solutions and precipitates are light colored.

The advantages of sodium hydroxide are the complete solubility of all compounds containing iodine, and more rapid hydrolysis of the acid-insoluble products. This results in a smaller weight of material precipitated by the addition of acid. Another distinct advantage attending the use of sodium hydroxide is the small volume of the solution. 45 kilos of fresh thyroid material can be treated in a total volume of 60 liters.

The only disadvantage associated with sodium hydroxide is the solubility of the dark impurities. This has been overcome by a method which will be described in detail. It is desirable to know the total iodine present in each sample of thyroid glands, and since sodium hydroxide furnishes such a medium, it was decided to



carry out the initial hydrolysis of the thyroid glands with sodium hydroxide. Both the volume of the solutions and the amount of sodium hydroxide were greatly reduced from the amounts originally used by Kendall. After several modifications, the following procedure was adopted:

2 kilos of flake sodium hydroxide are dissolved in 5 liters of water in a 35 liter nickel container. To this are added 22.5 kilos of fresh thyroid glands, and the total volume is made up to 30 liters. The solution is stirred for a few minutes until the alkali is thoroughly mixed with the glands. The nickel vessel is then heated without agitation by means of a boiling water jacket. The temperature is maintained between 95–100° for 24 hours, but the solution is not stirred after the initial thorough mixing with the alkali.

When the process is carried out in this way almost the entire amount of fat contained in the glands rises to the surface and can be separated without difficulty. It is not removed during the heating as it prevents evaporation of the water. After the solution has been heated for 24 hours, it is drawn off through a siphon into a large stoneware crock containing 20 liters of water and 8 kilos of sodium chloride of technical grade. The solution is allowed to cool to room temperature; the layer of fat on the surface is then removed, together with the uppermost layer of the sodium soap of fatty acids which were saponified during the treatment with alkali. A few kilos of ice are added to the solution which is then made acid with commercial hydrochloric acid. Sufficient fatty acids are present to cause the precipitate to rise to the surface.

Separation of the insoluble material is made more complete by the sodium chloride resulting from the neutralization of the alkali and by the sodium chloride which was added. After 3 or 4 hours the solution is siphoned out of the crock, care being exercised that none of the flocculent precipitate is lost. The portion that remains in the crock is filtered through a suction funnel on a pad of infusorial earth.

The precipitate is placed again in the nickel container and dissolved in 10 liters of 5 per cent sodium hydroxide and the solution is heated for 18 hours. From 400 to 500 gm. of crystalline barium hydroxide, depending on the amount of fatty acids present,

are dissolved in hot water and added to the solution which is heated for 3 hours after the addition of the barium hydroxide. The barium hydroxide precipitates all of the fatty acids which are present and also carries out of solution a large amount of the black, tarry impurities.

Thyroxine is not precipitated by the barium at this point, because only a small amount of barium hydroxide remains not precipitated by the tarry material and the fatty acids, and also because barium hydroxide does not precipitate thyroxine in a solution containing products of hydrolysis and this concentration of sodium hydroxide. If an insufficient amount of barium hydroxide is added, the solution is difficult to filter, but if the tarry material has been completely precipitated as insoluble barium salts, the solution filters easily on a large Buchner funnel.

The filtrate is made acid with hydrochloric acid which produces a small flocculent precipitate, Fraction A. This is allowed to settle to the bottom of the container for a few hours and is then removed by filtration. By these two steps, which are not time-consuming, the thyroxine is concentrated in a small fraction, and from control experiments with English desiccated thyroid in which material has been treated both by Harington's method and by the method outlined, it has been shown that the loss of thyroxine from this hydrolysis is not significant. For the completion of the hydrolysis of thyroxine from the protein and in order to destroy the interfering compounds as much as possible, the precipitate is treated with a high concentration of barium hydroxide. In Kendall's original method, the partially purified thyroxine was repeatedly treated with 10 per cent barium hydroxide. The fraction which was insoluble in this solution was subsequently crystallized in the form of its sodium salt. Harington suggested the use of stronger barium hydroxide solution.

Fraction A is dissolved in a total volume of 500 cc. of water containing from 200 to 250 gm. of crystalline barium hydroxide. Ammonia is used to assist solution. After being heated for from 18 to 20 hours, the solution is filtered hot and the barium-insoluble material is decomposed with sodium hydroxide and sodium sulfate. The barium hydroxide solution is made acid with hydrochloric acid and the precipitate is redissolved in sodium hydroxide and reprecipitated to free it from barium. Thyroxine will not separate

from a solution of this precipitate after it has been dissolved in dilute alkaline alcohol and has been made acid with acetic acid. With some samples of thyroid material, however, fractional precipitation with ammonium chloride may separate thyroxine which can be further purified and finally crystallized from alcohol.

The sodium hydroxide-sodium sulfate solution of the barium-insoluble material is made acid and a heavy, black, tarry precipitate separates from the solution. This material is dried and weighed.

In the description of the hydrolysis of thyroid proteins with barium hydroxide, Harington (9) pointed out that the use of this alkali produces light colored solutions which contrast favorably with the similar solutions of thyroid material secured by hydrolysis with sodium hydroxide. The appearance of the solutions and precipitates which are obtained by the use of sodium hydroxide is not encouraging, but a simple treatment has been found which results in an almost quantitative separation of the black, tarry impurities from the thyroxine and other colorless compounds which are present. This separation is based on the insolubility of the sodium salt of the black impurities in cold neutral 95 per cent alcohol. The details of the separation are as follows: 1 part of the dried acid-insoluble material precipitated from the solution of the barium-insoluble fraction in sodium hydroxide and sodium sulfate, is dissolved in about 150 parts of 95 per cent alcohol which also contains a small amount of hydrochloric acid. The solution is cooled to 10°. Finely powdered sodium hydroxide is now added from the end of a spatula, with constant stirring. Each addition of the hydroxide causes turbidity and the neutralization of the hydrochloric acid is continued until red litmus paper turns faintly blue. At this hydroxyl concentration the sodium salt of the black, tarry impurities is almost entirely insoluble and settles out as a brown precipitate, which is easily removed by filtration or centrifugation. The separation of the impurities is made more complete by the addition of 3 or 4 gm. of sodium hydroxide and treatment of the solution with carbon dioxide. This removes the brown impurities from the solution. The sodium carbonate and precipitated material are filtered out and the light colored filtrate is acidified with acetic acid. This is heated on a steam bath for from 6 to 8 hours under conditions of minimal evaporation.

Thyroxine if present, separates as a white, crystalline material. A small amount of thyroxine, from 100 to 200 mg., may sometimes be secured by retreatment of the neutral alcohol precipitate and of the mother liquor of the thyroxine, with 40 per cent barium hydroxide.

By the method outlined, many samples of thyroid glands from hogs have been treated and the products have been divided into various fractions. Certain definite conclusions can be drawn from this work regarding the variations in the total iodine and in the distribution of the iodine compounds.

*Total Iodine.*—The total iodine content of 45 kilos of fresh thyroid glands from hogs has been determined in samples which have been collected during the first few days of each month during the last 24 months. Seidell and Fenger (25) and Fenger (8) have previously reported a seasonal variation in the iodine content of thyroid glands removed from beef, sheep, and hogs that were killed in Chicago. Our results confirm their work. There is a marked seasonal variation which amounts to almost 300 per cent.

The number of animals necessary to furnish the thyroid material (about 5000 are required for each 45 kilos) prevents control of the diet. It seems probable, however, that iodides were not furnished the animals, and that the variations in the iodine in the thyroid glands are caused by one or both of two factors: variations in the iodine of the food, and variations in the temperature. Fenger has suggested that the seasonal variations in the iodine content of the thyroid gland are due principally to changes in the temperature. These results strengthen this hypothesis but variations in the iodine content of the diet cannot be excluded by this type of experiment.

*Inorganic Iodide.*—From the results of Nürnberg (17), Weir (27), Meyer (16), Kendall (14), and others, it appears certain that the normal thyroid gland does not contain inorganic iodide. Furthermore, sodium hydroxide does not break off any organically bound iodine from the proteins of the normal gland. Goiters removed from patients who have recently received compound solution of iodine contain iodides which are found as sodium iodide after hydrolysis with sodium hydroxide.

*Iodine-Containing Compounds Soluble in Acid.*—The percentage of the total iodine which is soluble in acid after alkaline hydrolysis

is remarkably constant for normal glands. It is significant, however, that this percentage can be made to vary. The addition of iodides to the diet of sheep for several months markedly increased the percentage of iodine compounds insoluble in acid after hydrolysis. The other extreme is found in glands removed from patients with exophthalmic goiter. In this disease, the iodine in the compounds insoluble in acid after hydrolysis of the gland approached zero. This may be due principally to the small amount of iodine present of which a higher percentage would apparently be soluble, but it appears highly probable that the percentage of the total iodine in the acid-insoluble material was less than that which occurs in the normal gland.

*Iodine-Containing Compounds Insoluble in Acid.*—The chemical properties of the acid-insoluble iodine-containing compounds and the stability to further alkaline hydrolysis become apparent from determination of the percentage of the total iodine which is separated in the various fractions. After the initial hydrolysis of the thyroid proteins the acid-insoluble products are again treated with 5 per cent sodium hydroxide. This does not destroy thyroxine, but it results in rendering acid-soluble about 13 per cent of the total iodine which was insoluble in acid after the first hydrolysis. A further fractionation of the iodine compounds is brought about by the addition of barium hydroxide to the second sodium hydroxide solution. A variable percentage of the total iodine is precipitated by the barium hydroxide. This fraction is found to vary not only with the season but with the condition of the thyroid gland. One group of thyroid glands from sheep that had been fed potassium iodide for several months contained scarcely any iodine compounds which were precipitated by barium hydroxide. In samples of thyroid glands from hogs, particularly in the winter months, iodine compounds are present which are precipitated by barium hydroxide. The iodine-containing compounds which are stable to alkali and which are insoluble in acid after the second treatment by sodium hydroxide, and are not precipitated by dilute barium hydroxide, are found to be approximately 20 per cent of the total iodine. (See Table I and Fig. 1.)

*Iodine-Containing Compounds Insoluble in 40 Per Cent Barium Hydroxide.*—Thyroxine and other iodine compounds are separated by means of concentrated barium hydroxide. All of the iodine-

containing compounds which are insoluble in concentrated barium hydroxide solution cannot be separated as thyroxine.

In twenty-three experiments the average percentage of the total iodine which was insoluble in 40 per cent barium hydroxide

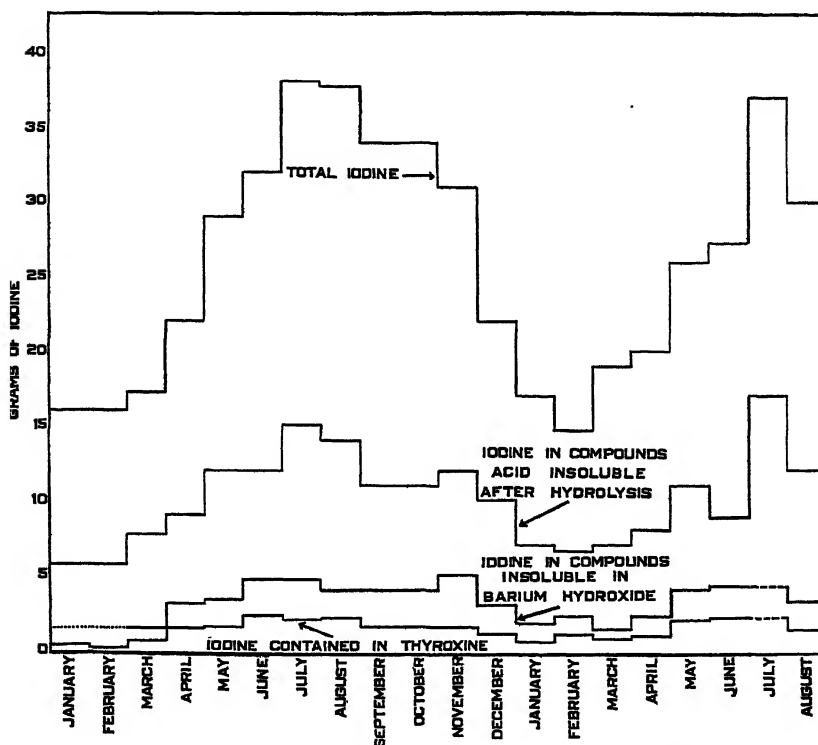


FIG. 1. The four curves show the weight of iodine which was present in the various fractions after alkaline hydrolysis of 45 kilos of fresh hog thyroid glands. Experiments were carried out between January, 1927, and August, 1928.

was 12. The average percentage of the total iodine separated as thyroxine was 5; this is a yield of 42 per cent of the iodine compounds insoluble in barium hydroxide which could be separated as thyroxine. The highest yield was 66 per cent and was obtained from glands which were removed from animals that had received

TABLE I.  
Iodine in Hydrolyzed Fractions of Thyroid Material.\*

Experiment No.	Date.	Weight.	Source of material†	Total iodine.	I in acid-insoluble fraction A.	I in fraction A soluble in dilute Ba(OH) <sub>2</sub> fraction	I in fraction C in acid.	Fraction D, insoluble in 40 per cent Ba(OH) <sub>2</sub> .	Fraction E, soluble in 40 per cent Ba(OH) <sub>2</sub> .	I in fraction D.	I in fraction E.	Thyroxine.	I insoluble in acid after hydrolysis, fraction A.	I in fraction A after addition of dilute Ba(OH) <sub>2</sub> fraction	I in fraction C in acid.	I insoluble in 40 per cent Ba(OH) <sub>2</sub> fraction D.	I soluble in 40 per cent Ba(OH) <sub>2</sub> fraction E.	I in form of thyroxine.
	1926																	
1	Nov. 26	45	H	20.7	8.0	3.5												
2	Dec. 3	45	B	25.5	9.7	3.1												
3	" 16	45	S	14.4	3.8	1.1												
	1927																	
4	Feb. 1	1.36	"	2.9		1.61		1.150	0.350	0.443	0.378	0.450				15	13	10
5	" 7	45	H	15.61	5.7	2.6						0.330						1.4
6	Mar. 4	1	DS	5.6	3.06	1.8			0.600			0.440				11		5.2
7	Feb. 9	1	"	5.0	2.5	0.365												
8	" 9	8.86	S	4.0	1.9	0.636												
9	" 19	2.72	"	0.778		0.171												
10	Mar. 10	45	H	15.5	5.6			4.3	0.900		0.291	0					1.9	0
11	" 2	1.2	DH	2.9	1.38	0.535		2.1	0.700		0.082	0					2.8	0
12	" 22	7.70	S	1.4	0.744	0.298												
13	" 22	2.26	"	0.184	0.059	0.045												
14	Apr. 4	1	DS	5.9	2.4	1.6		2.05	0.550	0.762	0.183	0						
15	" 6	45	H	17.7	7.7	2.8		4.9	1.55	1.44	0.436	0.700				13	3.1	0
16	" 8	0.325	"	0.402	0.122	0.092										8.1	2.4	2.6
17	" 30	45	"	21.77	9.29		4.93	8.5	1.5	3.04	0.371	12.21						
18	May 23	45	"	29.92	9.45		5.29	11.19	2.48	3.64	0.615	2.85				14	1.7	6.7
19	June 2	45	"	28.88	11.95		6.02	9.65	1.93	3.43	0.433	2.41				12	2.0	6.8
20	" 19	45	"	30.0	15.41		7.59	14.73	2.16	4.19		2.83				21	1.4	5.5
21	July 6	1.35	"	0.502	0.226											25	14	6.1
22	" 12	45	"	32.05	12.10	10.20	6.92	22.48	3.45	4.71	0.886	3.43				15	2.7	7.0

\* Per cent of total I in gland.

23	July	13	0.963	H	0.921	0.254	7.93	15.02	3.52	4.43		28	40	23	13	1.8	4.5
24	"	13	0.771	"	0.343	0.169	13.87	13.40	1.79	5.25	0.661	2.37	31	21	14	1.0	6.6
25	"	14	45	"	35.04	17.73	11.41	7.87	18.20	2.55	4.46	0.398	34	33	12	1.4	5.0
26	"	25	45	"	36.63	12.39	12.77	7.51	14.17	2.92	3.92	0.544	39	33	19	1.8	6.1
27	Aug.	1	45	"	38.25	14.37	11.30	6.97	11.70	3.08	3.79	0.711	37	30	17	2.0	5.3
28	"	29	45	"	37.59	14.01	11.30	5.85	11.70	3.08	3.79	0.711	44	43	11	2.0	5.3
29	"	31	45	"	34.17	15.18	14.81						48				
30	Sept.	7	2.7	"	3.99	1.90	1.30	1.07	2.8	0.250	0.285	0.102	46	38	32	8.4	3.0
31	"	21	2.7	"	3.38	1.57	1.30	1.07	2.8	0.250	0.285	0.102	46	38	32	8.4	3.0
32	"	21	1.0	DS	1.76	1.12	0.991	0.766	0.230	0.590	0.085	0.092	64	56	44	3.6	5.2
33	"	28	1.64	S	0.364	0.166	0.095	0.031					46	26	8.5		0
34	"	28	1.11	"	0.265	0.102	0.088	0.035					40	35	14		
35	"	28	1.86	"	0.233	0.099	0.052	0.021					42	22	9.0		
36	Oct.	4	45	H	28.69	10.78	9.91	5.71	13.2	3.42	4.24	0.774	38	35	20	15	2.6
37	"	10	1.0	DS	1.73	1.04	0.887	0.482	1.47	0.500	0.201	0.054	60	51	28	12	3.1
38	"	14	0.850	S	0.292	0.112							38				0
39	"	14	1.07	"	0.218	0.084							39				
40	Nov.	2	45	H	34.45	10.72	8.97	6.06	12.59	3.30	4.22	0.729	31	26	18	12	2.1
41	Dec.	8	45	"	31.40	12.03	10.71	6.50	12.47	3.01	5.24	0.742	38	34	21	17	2.3
42	Jan.	3	45	"	22.23	9.90	8.77	4.66	8.51	2.32	2.83	0.780	45	39	21	13	3.5
43	Feb.	7	45	"	16.71	7.03	6.05	3.31	6.55	2.66	1.78	0.584	43	36	20	11	3.4
44	Mar.	2	45	"	14.71	6.67	5.41	3.44	6.11	1.55	2.26	0.328	45	37	23	15	2.2
45	"	28	45	"	18.36	7.38	3.83	3.18	3.55	1.10	1.29	0.268	39	21	12	7.0	1.4
46	Apr.	4	45	"	18.86	5.38	5.39	2.08	8.40	2.45	1.73	0.701	29	29	16	9.1	2.8
47	"	7	45	"	16.11	5.44	3.74	2.18	3.25	2.25	1.14	0.591	34	23	14	7.0	3.6
48	May	11	45	"	19.90	8.26	5.80	3.98	7.5	2.55	2.43	1.20	42	29	20	12.0	3.9

\* The weights of iodine and percentage of the total iodine which is separated in the various fractions after alkaline hydrolysis of thyroid material. In the column which shows the weight of thyroxine isolated, 0 indicates that thyroxine was not present and could not be separated in crystalline form; blank spaces indicate that the separation of thyroxine was not carried through to the final stage.

† B, beef; D, desiccated; H, hog; S, sheep.



iodine. The lowest was 30 per cent; this was obtained from glands secured in January.

Although only from 30 to 40 per cent of the iodine in the barium-insoluble fraction can be separated as thyroxine, the alcoholic mother liquor from which thyroxine separates contains substances which have about the same solubilities as thyroxine except in regard to the solubility in alcohol. The mother liquor after thyroxine has been allowed to crystallize from solution for many hours may be retreated with 40 per cent barium hydroxide without material loss of iodine. A barium-insoluble fraction is obtained, but when this is again dissolved in alkaline alcohol and is acidified with acetic acid, thyroxine does not crystallize from the solution. There is conclusive evidence that the iodine present in this fraction is not in the form of thyroxine.

*Iodine-Containing Compounds Soluble in 40 Per Cent Barium Hydroxide.*—The iodine compounds soluble in 40 per cent barium hydroxide are divided into those soluble in dilute acid (this fraction contains inorganic iodides which are always present in the solution after the compounds have been heated with 40 per cent barium hydroxide) and those insoluble in acid. In twenty-one experiments the average percentage of the total iodine which was soluble in 40 per cent barium hydroxide and in acid was 7.5. In striking contrast to this average, Experiments 31 and 32 show 21 and 35 per cent, respectively, of the total iodine which was soluble in 40 per cent barium hydroxide and in acid. In twenty-five experiments the iodine compounds which are soluble in 40 per cent barium hydroxide but are insoluble in acid average 2.5 per cent of the total iodine.

Although this is not a high percentage of the total iodine it is equal to almost a half of the iodine separated in the form of thyroxine. Since the same procedure was followed in each of these experiments regardless of the source of the material, variations which are equivalent to 4 times the average figure become significant and they doubtless denote actual differences in the chemical composition of the iodine-containing compounds in the thyroid gland.

These investigations have shown that for the most part the iodine compounds soluble in 40 per cent barium hydroxide are not thyroxine. They cannot be regarded as decomposition prod-

ucts of thyroxine. The dried material contains little more than 30 per cent of iodine and its properties are such that it cannot be either crystallized or precipitated as a crystalline salt. Moreover, it has the power to prevent crystallization of added thyroxine.

*Thyroxine.*—In a series of twenty-three experiments the average yield of thyroxine was 5 per cent, the maximal yield was 10 per cent, and the minimal was 1.6 per cent. Since all of the thyroxine will not crystallize from a solution which contains impurities known to be present, it is impossible to assume that these yields represent the actual concentration of thyroxine in the material. The results, however, show variations in samples of thyroid material when treated by the same procedure. Furthermore, it is improbable that more thyroxine could be isolated by any other method. Even though all of the iodine insoluble in 40 per cent barium hydroxide is considered to be thyroxine, the average yield would be only 12 per cent. That this assumption is not justified is clearly shown by the chemical and physical properties of the iodine compounds in the mother liquor after separation of thyroxine.

When thyroxine is treated with 5 per cent sodium hydroxide, inorganic iodide is not present in the solution, even after it has been heated for 48 hours at 100°. It has been observed that inorganic iodides are present in each of the fractions after the first alkaline hydrolysis of thyroid material. This result indicates that the iodine compounds other than thyroxine are broken down with liberation of inorganic iodides.

*Thyroxine Content of Desiccated Thyroid.*—In Experiments 6, 7, 11, 14, 32, and 37 desiccated thyroid was used in place of fresh thyroid material. From only one sample was thyroxine isolated. Some of these preparations were made in England, although the source of the material was not known. Other samples were prepared in the United States. Since some thyroxine was isolated from all except one sample of fresh thyroids from hogs, it is significant that from practically all samples of desiccated thyroid, thyroxine could not be isolated. Many other experiments not recorded in Table I were made on desiccated thyroid in which Harington's method was used, but thyroxine could not be separated.

Some of the samples of desiccated thyroid investigated contained

## 370 Iodine and Thyroxine in Thyroid Gland

more than the average amount of iodine. One sample contained 0.54 per cent of iodine and its physiological activity was shown by its effect on thyroidectomized swine. The hypothyroid condition was relieved in every detail and the animals gained in weight with the relief of the hypothyroid status. We are indebted to Drs. Caylor and Schlotthauer for the physiological testing of these products.

In 1896 Roos (23) showed that the physiological activity of thyroid material was destroyed by treatment with dilute potassium hydroxide, and that the physiological activity of the same sample of material was not destroyed by treatment with 10 per cent sulfuric acid. This result indicated that the active constituent of the thyroid was relatively much more stable to acid than to alkali. In order to determine whether treatment of some samples of desiccated thyroid with acid would destroy the physiological activity similar to the treatment with alkali, desiccated thyroid was treated with 10 per cent sulfuric acid for 5 hours and the iodothyron separated according to the method of Baumann. This fraction contained 4.6 per cent of iodine and was shown to possess typical thyroid activity. It relieved the condition of hypothyroidism in thyroidectomized swine as did the desiccated thyroid from which it was prepared. The iodothyron separated from 100 gm. of desiccated thyroid was then treated with 40 per cent barium hydroxide, but thyroxine could not be separated from the barium-insoluble material. We must, therefore, conclude that desiccated thyroid and iodothyron can possess the typical activity of thyroid material and yet from this material thyroxine cannot be isolated.

Experiments were then carried out to determine whether thyroxine is destroyed when it is heated in 5 per cent sodium hydroxide in the presence of protein or when it is boiled in 10 per cent sulfuric acid in the presence of protein. 500 mg. of pure crystalline thyroxine were dissolved in 500 cc. of 5 per cent sodium hydroxide and to this solution 25 gm. of gelatin were added. The solution was heated for 24 hours. 44 mg. of organically bound iodine were in the acid-soluble fraction. The acid-insoluble material was dissolved in alkaline alcohol to which acetic acid was added; 310 mg. of crystalline thyroxine were recovered. This result clearly shows that thyroxine is not destroyed by the action of

sodium hydroxide in the presence of gelatin. After it had been shown that pure crystalline thyroxine which had been mixed with gelatin could be recovered after treatment with 5 per cent sodium hydroxide, it seemed probable that the inability to separate thyroxine from iodothyronin was due to the effect of acid on thyroxine in the presence of protein material. This possibility was investigated by the hydrolysis of 25 gm. of gelatin in 500 cc. of 10 per cent sulfuric acid to which 500 mg. of crystalline thyroxine had been added. The solution was boiled for 5 hours; it was then cooled and filtered. 38 mg. of iodine were in the acid-soluble fraction, although inorganic iodides were not present. The acid-insoluble precipitate weighed 445 mg. and from this 350 mg. of crystalline thyroxine were separated. Thyroxine was also heated in 10 per cent sulfuric acid with alanine and with a mixture of alanine and glucose. In neither of these experiments was the thyroxine appreciably decomposed, and it was recovered unchanged for the most part.

Similar experiments were also carried out with 25 gm. of gum acacia in 500 cc. of 5 per cent sodium hydroxide to which 500 mg. of thyroxine had been added. The thyroxine was recovered in about the same yield as from a solution of gelatin. The same amount of thyroxine added to 25 gm. of gum acacia in 500 cc. of 10 per cent sulfuric acid showed that this solution did not destroy the thyroxine, although some of the material appeared to be carried down in the fraction which is insoluble in neutral alcohol.

Iodine was not broken off by either the acid or the alkaline treatment and thyroxine was but slowly altered when boiled in a solution of gelatin, amino acids, or gum acacia with 5 per cent sodium hydroxide or with 10 per cent sulfuric acid. This stability of thyroxine is in striking contrast to the high percentage of the total iodine which is soluble in acid after hydrolysis of thyroid material. Treatment has not been found by which the products soluble in acid subsequently become acid-insoluble and it appears certain that this fraction of the iodine-containing compounds cannot be derived from thyroxine by any method of degradation of thyroxine itself. The ether linkage between the two benzene rings of thyroxine requires drastic treatment before it can be broken, and it has been repeatedly shown that 5 per cent sodium hydroxide cannot decompose the thyroxine molecule with libera-

tion of iodine. Quite apart from any physiological interpretation, it would appear that thyroxine must represent an intermediate form in the elaboration of the physiologically active compound, which is probably derived directly from thyroxine.

*Physiological Activity of Hydrolyzed Products of the Thyroid.*—Through the work of many investigators, it has been clearly established that every physiological effect of desiccated thyroid can be produced with thyroxine alone. This, however, is but a partial answer to the problem concerning the relationship between the activity of the thyroid gland and the iodine compounds which are present in the gland. After alkaline hydrolysis of the thyroid proteins, the products which are soluble in acid do not possess any demonstrable physiological activity. The acid-insoluble products which contain from 35 to 50 per cent of the total iodine possess qualitatively the same activity as desiccated thyroid but quantitatively they are much less active. A question of great significance concerns the relative activity of thyroxine when compared to desiccated thyroid.

Hunt (10), Cameron and Carmichael (5), and Boothby, Sandiford, Sandiford, and Slosse (4) and others have compared the activity of desiccated thyroid and of thyroxine on the basis of their iodine content. Hunt found that with the acetonitrile test desiccated thyroid was  $1\frac{1}{2}$  times as active as thyroxine. Cameron and Carmichael investigated the relative activity of desiccated thyroid and thyroxine by their effects on the growth and hypertrophy of certain organs in young rats. Their results are difficult to interpret, but it is clearly evident that all of the physiological activity of the desiccated thyroid is not due to the thyroxine in the material. Boothby, Sandiford, Sandiford, and Slosse compared the influence of desiccated thyroid and thyroxine on the basal metabolic rate of a patient with myxedema. They gave 3.9 gm. of desiccated thyroid which contained approximately 14 mg. of iodine. This is equal to the iodine contained in approximately 20 mg. of thyroxine and it produced an increase in basal metabolism which was almost the same as that produced by the injection of 10 mg. of thyroxine. In this type of experiment there is uncertainty regarding the amount of desiccated thyroid absorbed and the amount of thyroxine which is excreted in the bile and is not utilized. For the present, however, it is the best result available and it indicates that

50 per cent of the iodine in the desiccated thyroid was not present as thyroxine.

From the acetonitrile test we can conclude that desiccated thyroid is more active than thyroxine, and from the effect on the basal metabolic rate that thyroxine is somewhat more active than desiccated thyroid; but even accepting the latter conclusion, it is impossible to explain the physiological activity of all samples of desiccated thyroid by the thyroxine which can be isolated and it is impossible to account for the physiological activity of many samples of desiccated thyroid which do not contain any thyroxine which can be isolated in crystalline form. It seems highly probable that the acid-insoluble iodine compounds which are not thyroxine may be formed from some derivative of thyroxine which possesses the physiological activity of thyroxine and is stable to acid hydrolysis but is unstable to alkalis.

*Quantitative Relation between the Physiological Activity of Desiccated Thyroid and Thyroxine.*—Although qualitatively thyroxine is the full equivalent of desiccated thyroid and although quantitatively when the two products are compared on the basis of their iodine content there is not a great divergence in physiological activity, still when a quantitative measure of the amount of thyroxine which can be isolated from the gland is taken into consideration, it at once becomes apparent that the increase in basal metabolism which is produced by desiccated thyroid cannot be due only to the influence of the thyroxine which can be isolated in crystalline form. Since, however, there is no difference qualitatively, it is necessary to assume that there is a substance present in the thyroid gland which is not thyroxine but which has the same action, qualitatively, as thyroxine; quantitatively it is more active than thyroxine. This substance which reacts similarly to thyroxine is designated "active thyroxine." It is impossible to determine the quantitative relationship between the physiological activity of the thyroxine and active thyroxine. Since at least 50 per cent of the iodine in the thyroid gland is present in some form of combination which cannot be either thyroxine or active thyroxine, active thyroxine would appear to possess at least twice the activity of thyroxine. The amount of thyroxine actually isolated does not exceed 14 per cent of the total iodine, and the average amount found throughout this investigation was 5 per

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cent. Since thyroxine has been shown to possess but little if any more activity than desiccated thyroid, from 86 to 95 per cent of the physiological activity of thyroid material is due to active thyroxine.

Data of this nature do not permit a prediction concerning the chemical nature of active thyroxine, but it seems highly probable that thyroxine represents an intermediate stage in the elaboration of active thyroxine which is the actual functioning constituent of the thyroid gland. The results of many investigators, among whom may be mentioned Oswald (19), Baumann (2), Tambach (26), Hutchison (11, 12), von Cyon and Oswald (7), Baumann and Roos (3), Kendall (14), and others, have shown that the iodine-containing compounds are constituents of the protein molecule and do not exist in the free form. However, the combination of thyroxine within the protein molecule is not sufficient to explain the difference between thyroxine and active thyroxine. Hydrolysis of the protein by alkali should result in the isolation of far more than from 5 to 15 per cent of the total iodine in the form of thyroxine if active thyroxine were actually thyroxine as isolated in the crystalline form. The conclusion appears to be justified, therefore, that the thyroxine molecule is altered in some way before it possesses the physiological activity of active thyroxine and of desiccated thyroid. This alteration can be brought about by the tissues in an organism which does not possess a thyroid gland, and although it is impossible to determine the chemical nature of active thyroxine, there is much to suggest that the conversion of thyroxine to active thyroxine is brought about by the addition of a second hydroxyl group, probably to the outer benzene ring of thyroxine. The striking effect of hydroxyl groups in the benzene ring in biological oxidation has been well established through investigations of quinol, chlorogenic acid (18), and phenols shown by the work of Robinson and McCance (22), McCance (15), Pugh and Raper (21), and many others in a study of the action of tyrosinase. Tyrosine is stable to alkali; dihydroxyphenylalanine is unstable to alkalis. A similar relation may explain the instability of active thyroxine to alkalis. \*

If the relation between the physiological activity of the thyroid gland, thyroxine, and active thyroxine at least in part accords with the hypothesis which has been given, it is not difficult to explain

the absence of thyroxine which can be isolated in crystalline form from some samples of desiccated thyroid which possess the typical physiological activity of thyroid material. In such samples of desiccated thyroid, it is highly probable that the thyroxine which was present in the thyroid glands before they were removed from the organism has been converted into its unstable or active form either before or during the desiccation. Preliminary experiments have indicated that it is possible to decrease materially the percentage of the total iodine which is present in the form of thyroxine merely by suspending minced thyroid material in tap water for a few days. Further work is now being carried out with thyroid material in order to study the chemical properties of thyroxine as it occurs in the proteins of the thyroid gland and in particular in its relation to active thyroxine.

#### SUMMARY.

The products of alkaline hydrolysis of the thyroid material may be separated into acid-soluble and acid-insoluble fractions. The assumption that the iodine in the acid-insoluble fraction is all present in the form of thyroxine has been shown to be incorrect. Not more than from 20 to 30 per cent of the iodine in this fraction is in the form of thyroxine.

Hydrolysis of thyroid material indicates that there are many iodine compounds present, all of which are combined to the protein. Inorganic iodides are not present in the normal thyroid gland. Sodium hydroxide does not break off iodine in the inorganic form from the proteins of the normal thyroid gland. Inorganic iodides are present in solution after alkaline hydrolysis of goiters which were removed after administration of iodides. A comparison of sodium hydroxide and of barium hydroxide for the hydrolysis of thyroid proteins has shown certain advantages of each. A modification of Kendall's original method is given. There is about a 300 per cent variation in the total iodine content due to seasonal variation. The iodine contents of several fractions of the products of hydrolysis are given.

The iodine present in the form of thyroxine in fresh hog thyroid glands is not more than from 0.0 to 14 per cent of the total iodine. In twenty-three experiments the average yield of the total iodine in the form of thyroxine was 5 per cent. Desiccated thyroid and



iodothylin which possess typical thyroid activity may not contain any thyroxine which can be separated in crystalline form.

Thyroid material is much more stable to acid hydrolysis than to alkaline hydrolysis. It is impossible to explain the physiological activity of fresh or desiccated thyroid by the thyroxine which can be separated in crystalline form. Thyroxine appears to be an intermediate form of the active constituent of the thyroid. It is suggested that thyroxine must be converted into another form, "active thyroxine," before it can exert its physiological activity.

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## THE DETERMINATION OF MORPHINE.\*

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The estimation of morphine in biological material is difficult and tedious. Many methods have been advocated, not one of which has received the approval of successful employment by a considerable number of investigators. In the preliminary stages of projected work upon the behavior of morphine in the metabolism, it was necessary to subject existing methods to critical study for the purpose of identifying, and if possible of correcting, their inherent defects. It is too much to hope that a method for morphine shall be developed as reliable as those for inorganic substances; but it should be possible to discover what features in existing methods are most apt to give loss or contamination, and also to introduce some means of testing the reliability of an estimation after it has been made. The result of our work has been the selective elaborations of a series of methods for various classes of materials, which avoid such pitfalls as we have recognized, and with which we have secured satisfactorily consistent and reliable results.

All methods for determining morphine consist of two parts: first, isolation of the alkaloid in a state of relative purity; second, measurement of its amount.

In the early work on the qualitative recognition of morphine, suggestions are to be found upon which later quantitative methods have been built. Thus Lassaigne (1) removed the alkaloid by extraction in alcohol, and purified it by lead precipitations. The two classical procedures of Stas-Otto and Dragendorff include preliminary purification by the successive use of dilute acid and acid-

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alcohol, followed by alkaline extraction with amyl alcohol; and this in turn with dilute acid. Final purification of the watery solution was accomplished by means of charcoal, ether extraction, or the precipitation of contaminants in alcohol, and efforts were made to crystallize the free base. Since these usually failed, various color reactions were employed to identify the morphine; ferric chloride as determined by Lassaigne, nitric-sulfuric acid by Erdmann (2), Cloetta (3), Eliassow (4), Froehde's reagent by Kauzman (5) and by Landsberg (6).

In detecting morphine in urine, Notta and Lugan (7) used lead subacetate to remove impurities, finally isolating the morphine in amyl alcohol. Donath (8), however, with urine which must have contained morphine, obtained negative results by this method.

#### *Quantitative Work with Morphine.*

The first quantitative method was that of Tauber (9), who proceeded by removing all the impurities possible with successive heat coagulation and lead subacetate precipitation, finally crystallizing free morphine. The method, also used by Faust (10) in his work on the excretion of morphine in normal and tolerant dogs, is as follows: The material (100 to 200 gm.) mixed with about 4 times its volume of water, and slightly acidified with acetic acid, is boiled vigorously until the filtrate is clear. The coagulum is filtered off, washed with dilute acid, and the liquid obtained completely precipitated with lead subacetate. The lead precipitate is washed with water, then with alcohol. The solution, freed from alcohol and lead, is evaporated, some impurities still remaining are precipitated by alcohol, and the morphine finally crystallized from a  $\text{NaHCO}_3$  solution of small volume and weighed, a correction being made for the solubility of the alkaloid. If still impure, the lead precipitation is repeated. Control experiments gave very good recoveries, 90 to 95 per cent.

Tauber's method was considered unsafe by Wachtel (11), Zahn (12), Valenti (13), and possibly others on account of occasional contamination of the morphine with calcium carbonate; by Cloetta (14) because the final crystallization of morphine was incomplete if the original tissues contained much lipid. Modifications, combining the precipitation process of Tauber with the older extraction methods, began to be proposed. Cloetta's (14) modifica-

tion, including an isobutyl alcohol extraction, suggests a refinement of the scheme of Notta and Lugan, together with a final exclusion of impurities by solution of the alkaloid in a mixture of ethyl alcohol, ether, and chloroform. Grüter (15), working with chick embryos, used a similar but more elaborate technique, based on the Tauber method.

Both Rübsamen (16) and Wachtel (11) first removed the morphine by treating the tissue with alcohol. Rübsamen further clarified his solution by a heat-acid-salt coagulation, Wachtel by a lead subacetate precipitation. The final morphine determinations were novel and more accurate than the older precipitation of the free base. Wachtel measured the oxidation of morphine to pseudomorphine, Rübsamen the acidity due to the progressive hydrolysis of morphine salt, when the hydrolytically formed free base was continuously removed in chloroform.

*Retention of Morphine in Precipitates.*—All of these methods include in one form or another the precipitation procedures which underlie the Tauber process, as opposed to purely extractive means of isolation. They have always seemed to give fairly good results (recoveries ranging from 60 to 90 per cent on controls), particularly when large amounts of morphine were employed. It seems possible, however, that a loss of morphine through adsorption on the precipitated impurities might be a fundamental error in any of these processes. This we found to be true of precipitates formed by the lead subacetate clarifications of morphine solutions, but not true of the coagula produced by boiling with dilute acid, when these coagula were persistently washed—a very tedious operation, however.

In order to test the retention of morphine by such precipitates under what appeared to be reasonable conditions, 100 gm. of ground beef muscle were mixed with 100 mg. of morphine, suspended in 1 liter of water, and acidified slightly with acetic acid. This mixture was boiled vigorously until coagulated, filtered hot, and the coagulum washed with 0.5 per cent acetic acid until a test with Froehde's reagent was negative on 5 cc. of the filtrate (evaporated to dryness). The washing occupied 3 days but used only about a liter of fluid, added in small portions. The coagulum was then ground in sand, digested with 1500 cc. of warm alcohol containing 0.5 per cent hydrochloric acid, and a large proportional

part of the liquid was then filtered off. This was treated as described later under our proposed method for muscle and yielded a final solution which contained nothing precipitable by silicotungstic acid (indicating less than 1 mg. of morphine) and gave no color reaction with *p*-dimethylaminobenzaldehyde<sup>1</sup> (indicating less than 0.05 mg. of morphine).

We believe, therefore, that morphine is not adsorbed by the heat coagulated proteins.

Lead subacetate was added to the filtrate from this coagulum and then about 1 gm. of NaCl to precipitate the colloid formed. A heavy flocculent precipitation was complete after 2 days standing. This was washed with water until the washings were negative to Froehde's reagent, then with alcohol for several times after a test on 5 cc. of the alcohol washings gave a negative Froehde reaction (the alcohol washings contained 1.9 mg. of morphine not removed by the water, as determined by our subsequently described methods). These washings took 2 days. The lead precipitate was next suspended in 1200 cc. of alcohol, this made acid to Congo red with sulfuric acid, and allowed to stand then overnight. A large aliquot was filtered off and analyzed for morphine by our procedure. 3.4 mg. of morphine were obtained in this way, which also responded characteristically to both the Froehde and *p*-dimethylaminobenzaldehyde tests.

A similar experiment was made on the lead subacetate precipitate from urine. 50 mg. of morphine were added to 250 cc. of normal dog urine. The lead precipitate contained no water-insoluble, alcohol-soluble morphine. The finally washed precipitate, however, did contain 0.4 mg. of morphine base, isolated by the method given later for urine, and showing the characteristic color reactions of morphine.

From a solution of 150 mg. of morphine in 1 liter of water containing 0.3 gm. of peptone and a few drops of acetic acid, a pre-

<sup>1</sup> This reagent, suggested by Wasiicky (17), gives with morphine a brilliant red color, with pseudomorphine a faint, pale green. Other oxidation products of morphine and the substances commonly separated with morphine from tissues, form either no color or a faint brown tint. Thus the detection of morphine is possible in the presence of many impurities if other reacting alkaloids are not in question. Urine extracts, however, give a dark brown color which renders them unsuitable for this test.

precipitate was prepared by lead subacetate, but could only be filtered after the addition of 0.5 gm. of NaCl. This precipitate, washed with water and with alcohol as usual, contained 2.1 mg. of morphine base.

The coagulation of tissues by acid-alcohol may be regarded here as resembling that by heat and acetic acid. No better method than acid-alcohol extraction seemed available for removing the residual morphine from the coagula, so the recovery of morphine from the extract was determined as showing by difference the amount retained in the solid.

An experiment was made with fresh filtered egg white, to which morphine was added, and the protein coagulated by pouring the mixture with vigorous stirring into 25 volumes of absolute alcohol, containing 1 per cent acetic acid. The coagulum was filtered off, and extracted (unwashed) in a Soxhlet apparatus with alcohol for about 24 hours. Both the acid-alcohol filtrate and the alcohol extract were analyzed by the method later described for blood.

Had morphine been adsorbed by the precipitated protein, the amount of morphine in the fraction containing the coagulum should have been disproportionately larger than in the filtrate from it. The results, however, showed no such increased concentration of morphine, although the recovery of alkaloid totalled the amount originally taken.

*Experiment I.*—To 292 cc. of diluted egg white (18 gm. of dry solid) 194 mg. of morphine were added; 4 liters of acetic acid-absolute alcohol were used. The total weight of solution and precipitate equals 3675 gm. (in air). The acid-alcohol filtrate, 3555 gm. or 96.65 per cent of total weight, contained 190 mg. of morphine, or 98.0 per cent of the total morphine. The coagulum and adhering liquid weighed 123 gm. or 3.35 per cent of the total, and contained 5.0 mg. of morphine, or 2.60 per cent of the total morphine.

Total morphine recovered from acid-alcohol filtrate and alcohol extract of coagulum equals 195.0 mg. or 100.5 per cent.

*Experiment II.*—To 290 cc. of diluted egg white (representing 14.5 gm. of dry substance) 194 mg. of morphine were added; about 4 liters of acetic acid-alcohol were used. The total weight of solution and precipitate equals 3715 gm. (in air). The filtrate 3620 gm., or 97.56 per cent of the total, contained 195 mg. of morphine or 100.5 per cent of the total, while the coagulum and adhering liquid weighed 95 gm. or 2.56 per cent of the total, and contained 4.9 mg. of morphine, or 2.53 per cent of the total.

Total morphine recovered equals 199.9 mg., or 103.0 per cent.



These coagula, therefore, contained no appreciable accumulation of morphine, and no morphine not extractable by alcohol. Muscle tissue, however, similarly treated with acetic acid-alcohol, forms a coagulum richer in morphine than the surrounding solution. When part of this solution is filtered off, and analyzed for morphine by the proposed method for muscle, the results are low; while if absolute alcohol containing 0.5 per cent hydrochloric acid is employed, complete recovery in proportion to the fraction filtered off is obtained.

*Morphine Isolated from Muscle. Total Volume 500 Cc.*

	Muscle taken.	Morphine taken.	Volume of filtrate analyzed.	Morphine recovered in filtrate.	Recovery.
	gm.	mg.	cc.	mg.	per cent
Stirring with 1 per cent acetic acid-alcohol.....	30	31.8	425	20.0	74
Stirring with 1 per cent acetic acid-alcohol.....	30	31.8	425	21.4	79
Grinding in glacial acetic acid, then stirring into alcohol.....	20	31.8	425	21.4	79
Stirring with 0.5 per cent HCl-alcohol....	20	31.8	425	24.9	92

Conversely, when sodium acetate is added to such HCl-alcohol extracts, a gelatinous precipitate is thrown down, which by a similar method may be shown to contain morphine.

From blood, the coagulum formed by acetic acid-alcohol also retains a disproportionate amount of the morphine present in the system. The actual amount retained, however, is not constant, irrespective of the amount present, nor do repeated extractions of the coagulated blood proteins lead to complete recovery, as was the case with egg albumin. The cause of this retention of morphine is probably an adsorption, since the amount recovered may be considerably increased by the presence of substances capable of lowering surface tension (Table I). This may, however, be due simply to the formation of a more finely divided coagulum in these cases. Attempts to detect a difference in the surface tension of these solutions by the capillary rise technique were unsuccessful.

*Oxidation of Morphine during the Analysis.*—The several

methods which omit the precipitation of heavy metal salts nevertheless rely upon some form of coagulation process to remove the bulk of the tissue impurities. From the preceding experiments it appears that heat-acid coagulation is permissible, but tedious; acid-alcohol extraction is rapid as well.

Based upon the Stas-Otto procedure, the method of Van Rijn (18), which was followed in a general way by Tamura, avoids lead precipitates, but removes portions of the extracted salts, peptone,

TABLE I.

*Effect of Various Additions on Extraction of Morphine from Blood by Alcohol-Acetic Acid.*

20 mg. of morphine in 25 cc. of blood mixed with absolute alcohol, containing the substances described below. Total volume 500 cc.; morphine determined in a measured portion of filtrate and calculated for the total volume.

	Per cent recovery.
Neutral, boiled 30 min. under a reflux.....	42
1 per cent acetic acid.....	80
Neutral + 0.2 per cent Na oleate.....	84
1 per cent acetic acid + 0.2 per cent oleic acid.....	83
1 " " " " + 0.2 " " caprylic alcohol.....	86
1 " " " " + 2 " " amyl alcohol.....	88
Neutral + 0.2 per cent cetyl alcohol.....	79
1 per cent acetic acid + 0.2 per cent cetyl alcohol.....	88
1 " " " " + 1 " " " " .....	94

*etc.*, by a series of alcohol precipitations. The morphine is then extracted by amyl alcohol, dilute acid, and chloroform in turn, and finally precipitated as free base with petroleum ether.<sup>2</sup>

Losses are apt to attend so many hand-made extractions, and also the number of alcohol precipitations involved. Foaming of the solvents is a drawback.

Tamura (19) has minimized these losses by careful control of details, and recovered 80 to 90 per cent of the morphine, the final

<sup>2</sup> Van Rijn obtained good results, about 84 per cent recovery, from an injected rabbit, but von Kaufmann-Asser, testing the method on urine, not only lost all the morphine, but in addition obtained a positive blank.

determination of morphine being made according to Rübsamen (16).

Von Kaufmann-Asser's (20) method (for urine) eliminates several objections and simplifies the Van Rijn process by use of a continuous chloroform extractor for removing the morphine after a single alcohol precipitation of impurities. Recoveries from control experiments were 70 to 80 per cent.

A more recent development of the extraction technique is due to Takayanagi (21), whose method recalls somewhat the old gypsum procedure of Chandelon (22) for strychnine, *etc.*<sup>3</sup> After the morphine is removed from solid material with acid-alcohol and the alcohol replaced with water by evaporation, the aqueous acid solution is absorbed in sand, extracted with  $\text{CHCl}_3$  to remove impurities, then made alkaline, and dried. The dry sand and residue are again extracted with chloroform, furnishing sufficiently pure morphine for the final estimation, made by precipitation with phosphomolybdic acid. The recoveries reported by the author from control determinations on urine and on entire rat bodies are practically complete. The method is widely used.

Although avoiding certain errors of the precipitation methods, such extraction processes are still open to at least one objection, which rests upon the decomposition of morphine in neutral or alkaline solutions, particularly on evaporation.

In order to evaluate this error, pure morphine as the free base was recrystallized from water and dried at room temperature. Analyzed for  $\text{H}_2\text{O}$  of crystallization it showed (a) 5.81 per cent, (b) 6.08 per cent, (c) 5.91 per cent (theory for  $\text{C}_{17}\text{H}_{19}\text{NO}_3 \cdot \text{H}_2\text{O} = 5.94$  per cent). This material was placed in glass evaporating dishes, which, because of their size (125 cc.) were weighed by the usual counterpoise method. Known volumes of liquid were evaporated from the dishes, after each evaporation they were dried at  $120^\circ$  for a few minutes, and again weighed. The evaporation was done on a variable heat water bath and always considerably below the boiling point of the liquid in question. It was soon evident that the morphine underwent oxidation, the residue increased in weight (Table II), and eventually became brown and resinous in appearance. Finally, in order to determine the amount

<sup>3</sup> In view of Takayanagi's results, Chandelon seems to have been in error when he specifically excepts morphine as not determinable by his method.

of morphine remaining in the dishes, the residue was analyzed by our previously described (23) method for separating morphine from its oxidation products, which consists in extracting the alkaloid by chloroform-alcohol from a heavily buffered solution at pH 9.

While evaporation produced these progressive increases<sup>4</sup> in weight, continued heating of the dried residue, at 100° and even at 125°, caused no change.

The results show how easily morphine may be decomposed under conditions which frequently occur in the analytical laboratory. This decomposition was accelerated by a slight increase in the alkalinity of the solution (C, Table II). It is also shown to have been slower at first, with comparatively pure morphine in solution, and to have increased proportionally to the amount of oxidation products present (B, Table II). This may perhaps be explained by the fact that with pure morphine solutions much of the base crystallizes during the evaporation, but as the decomposition progresses, the soluble oxidation products hinder this crystallization, and the evaporating solution is more concentrated with respect to morphine. The aqueous solutions decomposed more rapidly than alcoholic or chloroform solutions, probably due to some effect of the higher temperature of evaporation.

Thus, the decomposition of morphine during evaporation of solutions of the free base may in a few hours reach 15 to 20 per cent of a reasonable analytical sample, as shown by a method for determining the existing morphine, easily accurate to 90 to 95 per cent. The conditions have been exaggerated, but the amount lost is startling. Several methods—notably that of Takayanagi—which include such alkaline evaporations give recoveries suggesting no such error. The reason for this is that the morphine oxidation products, as we have shown elsewhere, still retain many of the reactions of morphine. They are soluble in most morphine

<sup>4</sup> Unfortunately we did not perform similar experiments with acidified morphine solutions. It may be assumed, however, that no oxidation occurs, first, because repeated evaporations similar to those recorded showed no signs of brown coloration or resin formation, and second, because many times we have evaporated known amounts of morphine with dilute hydrochloric acid and with HCl-alcohol and subsequently determined the morphine, which showed no significant loss.

TABLE II.  
*Oxidation during Evaporation.*

A. Morphine in water solution.							
No. of evaporations.....	Start.	1	2	3	4	5	6
Volume evaporated (total cc.).....	0	100	200	250	300	400	500
Time of evaporation ( " hrs.).....	0	3½	6½	8½	11	13	16
Weight of residue (mg.).....	24.5	27.3	28.0	28.5	28.8	28.9	29.2

Morphine in final residue = 20.6 mg.

" decomposed = 3.9 " (16 per cent of original amount.)

B. Morphine in water solution.											
No. of evapora- tions.....	Start.	1	2	3	4	5	6	7	8	9	10
Volume evapo- rated (total cc.)..	0	35	135	185	195	245	345	445	495	595	645
Time of evapora- tion (total hrs.)..	0	2	5	7	7½	11	13	16	18	20½	22½
Weight of residue (mg.).....	66.5	66.3	66.4		66.7	67.7	68.2	69.0	69.9	70.2	71.2

Morphine in final residue = (a) 56.5 mg., (b) 58.0 mg.; average = 57.3 mg.

" decomposed = 9.2 mg. (14 per cent of original amount.)

C. Morphine in water solution + dilute NH <sub>4</sub> OH.				
No. of evaporations.....	Start.	1	2	3
Volume evaporated (total cc.).....	0	100	150	200
Time of evaporation ( " hrs.).....	0	5½	7½	9½
Weight of residue (mg.).....	23.1	24.3	25.1	25.6

Morphine in final residue = 18.3 mg.

" decomposed = 4.8 " (21 per cent of original amount.)

D. Morphine in ethyl alcohol.									
No. of evaporations.....	0	1	2	3	4	5	6	7	8
Volume evaporated (total cc.)...	0	50	100	150	200	240	265	290	315
Time of evaporation ( " hrs.)	0	1½	3½	5	7	8½	9½	10½	12
Weight of residue (mg.).....	29.1		29.7	30.5	31.0	31.7			32.2

Morphine in final residue = 28.3 mg.

" decomposed = 0.8 " (2.7 per cent of original amount.)

TABLE II—*Concluded.*

E. Morphine in freshly washed CHCl <sub>3</sub> .							
No. of evaporations.....	0	1	2	3	4	5	6
Volume evaporated (total cc.).....	0	50	60	85	110	130	160
Time of evaporation ( " hrs.).....	0	1	1½	3	4	5	6½
Weight of residue (mg.).....	49.4	50.7	51.1	51.3	51.2	52.1	52.5
Morphine determined in final residue = 48.2 mg.							
"    lost	= 1.2 " (2.4 per cent of original amount.)						

solvents, are precipitated by most morphine precipitants, in particular by the complex acids of tungsten and molybdenum, and they behave similarly to morphine in the diazo reaction of Lautenschläger (24).<sup>5</sup> Such oxidation products, when formed during an analysis for morphine, unless intentionally separated, are likely to follow the morphine and ultimately be determined as such, thus showing a nearly complete recovery in control determinations. No distinction, however, is possible by such methods between the oxidized morphine formed during analysis and any pre-existing in the material analyzed. With control determinations this is not a factor, but with material from morphinized animals it may defeat the very purpose of the experiment. It also seems possible (B, Table II) that the presence of morphine oxidation products coming from the original tissue would cause an even more rapid destruction of the morphine, to the point where a suitable color reaction (*e.g.* with *p*-dimethylaminobenzaldehyde) would be uncertain.

#### *Final Estimation of Morphine.*

Provided the isolation process has yielded pure morphine, almost any of the methods of final estimation are satisfactory. They are not all equally applicable, however, to both small and

<sup>5</sup> By a quantitative color comparison method based on Lautenschläger's reaction, Dr. Riegel has obtained identical results with morphine sulfate and pseudomorphine hydrochloride. A solution of morphine oxidized with hydrogen peroxide until it contained neither morphine nor pseudomorphine gave a color intensity equal to about one-quarter of that to be expected, if the total material precipitable from the solution by silicotungstic acid reacted as morphine.

large quantities of morphine, nor are they equally independent of the small amount of impurities which may yet accompany the isolated morphine. With very small amounts of morphine there appears to be no escape from colorimetric methods, in spite of their unreliability. With larger quantities, there is a great variety of acidimetric, gravimetric, oxidative, and optical procedures. In the presence of inescapable impurities, both oxidation and titration methods appear to us as uncertain. In the absence of other alkaloids, the optical rotation furnishes without doubt the most trustworthy estimation of morphine, especially since this may be checked by determinations in both acid and alkaline solution. Unfortunately, the quantity of morphine required is much larger than that usually being sought.

For our purpose, we have selected from among the gravimetric estimations the silicotungstic acid precipitation of Bertrand (25), not because of greater accuracy, as compared with other precipitants, but on account of the valuable check which may be made on the validity of the results.

Morphine silicotungstate may be dried and weighed on a Gooch filter, and then ignited in a muffle to a residue of constant weight. If the dried precipitate is pure, the ignition residue should be 70.3 per cent. For the commonly expected impurities, this value is quite different and allows a determination which has gone wrong through error, or because of some unusual tissue constituent,<sup>6</sup> to be noticed at once.

An account of the details for determining morphine as the silicotungstate has already been given elsewhere (23). For completeness, it is summarized here.

The morphine is finally isolated in a slightly acidified alcohol-chloroform solution. This is evaporated at a low temperature, the residual alcohol being finally replaced with a few cc. of water. The solution is filtered and washed through a tiny paper into a test-tube. It should be of small volume (certainly not over 10 cc.), and approximately 0.05 N with respect to hydrochloric acid. About 15 mg. of KCl are added, followed by a small excess of silicotungstic acid (10 per cent solution in water), the solution being tested by an extra drop after the subsidence of the precipitate. The contents of the tube are shaken vigorously and im-

<sup>6</sup> This is not as infrequent as might be imagined.

mersed in cold water until chilled. The precipitate is then brought into a weighed Gooch crucible, washed with cold water containing 2 cc. of concentrated HCl per liter, no more liquid than necessary being used. It is then dried at 120° (2 to 4 hours), weighed, ignited (in a muffle), and reweighed.

Somewhat at variance with the principles of this determination is the statement of Heiduschka and Wolf (26) that the composition of the silicotungstic acid precipitate may vary in the presence of 3 per cent or more HCl, and also that in the presence of 1 to 3 per cent NaCl, morphine uses just twice the amount of silicotungstic acid as in the presence of HCl. 1 mol of silicotungstic acid reacts therefore with 2 mols of morphine (instead of with 4 mols, as stated by Bertrand).

While the conditions of our precipitation are not exactly included in the cases discussed by Heiduschka and Wolf, they are sufficiently similar to be disturbing. We, therefore, precipitated known quantities of pure morphine under the circumstances discussed by these authors, and found that there was a slight deviation from the normal composition of the morphine silicotungstate as the concentration of HCl present was increased. This deviation was without significance at concentrations of 0.5 N acid (= 1.9 per cent hydrogen chloride) but became important thereafter. When NaCl was substituted for HCl, up to a concentration of 10 gm. of NaCl per 100 cc. of solution, no difference in the amount or composition of the precipitate was shown. This agrees with the formula of Bertrand, and not with that proposed by Heiduschka and Wolf for these conditions of precipitation. The data follow in Tables III and IV.

#### *Proposed Methods for Isolation of Morphine from Biological Material.*

In submitting the following methods we have tried to select a procedure which at least minimized many of the difficulties that have been discussed. In principle, it follows the extraction methods of the earliest workers. In practice, it has been necessary to vary the details with each kind of material taken for analysis. In general, the morphine is removed from tissues by soaking them in strongly acidified ethyl alcohol; methyl alcohol, in spite of the greater solubility of morphine, does not work as well. The



alcohol is replaced by water through careful evaporation, and the fat, *etc.*, removed in a cake of paraffin. The liquid is now strongly buffered and adjusted to a pH of 9.0, approximately the isoelectric point of morphine (Kolthoff (27)). It is then extracted in a liquid-liquid extractor with chloroform-alcohol, the solvent in

TABLE III.

*Effect of NaCl on Composition of Morphine Silicotungstate.*

Volume of solution precipitated = 10 cc., containing morphine as the purified hydrochloride, no HCl. Silicotungstic acid =  $\frac{1}{2}$  of amount calculated for complete precipitation, by Bertrand's formula.

	NaCl present in solution.	Weight of morphine silicotungstate.	Residue of morphine silicotungstate on ignition. Per cent of dried precipitate.
	<i>per cent</i>	<i>gm.</i>	
Calculated from weight of morphine used by formula of Bertrand (25).		0.1065	70.3
Calculated by formula of Heiduschka and Wolf (26).		0.1712	83.0
Results found.	1.0	0.1035	71.2
	1.5	0.1038	71.4
	2.0	0.1027	70.8
	2.5	0.1067	70.9
	3.0	0.1044	71.3
	3.5	0.1080	70.6
	4.0	0.1050	71.1
	4.5	0.1072	74.5
	5.0	0.1077	70.8
	6.0	0.1099	71.1
	7.0	0.1075	71.4
	8.0	0.1071	70.7
	9.0	0.1037	71.1
	10.0	0.1055	70.6

the extraction flask containing a little acid. The extraction of morphine at its isoelectric point is rapid and removes the morphine from oxidation products which may otherwise accompany it, as well as excludes other impurities. After the solvent is removed, the alkaloid is determined as the silicotungstate.

The advantages of the method are rapidity, simplicity, the exclusion of oxidized morphine, and the excellent check obtained by igniting the silicotungstate precipitate. Its disadvantage is a lack of sensitiveness, at least 5 mg. and better 10 mg. of morphine being needed for a reasonably accurate analysis.

*Determination of Morphine in Muscle.*

*Extraction of Tissue.*—From 25 to 50 gm. of finely ground muscle are mixed with 25 times their weight of absolute alcohol containing

TABLE IV.

*Effect of Increasing Concentrations of HCl on Composition of Morphine Silicotungstate.*

Volume of morphine solution precipitated = 10 cc., containing sufficient HCl to give the concentration indicated and in addition 15 mg. of KCl. Silicotungstic acid =  $\frac{2}{3}$  of amount calculated as necessary for precipitation of sample, 30.1 mg.

HCl concentration.	Weight of precipitate.	Residue on ignition.	Morphine calculated on oxide residus.
<i>mols per liter</i>	<i>gm.</i>	<i>per cent</i>	<i>mg.</i>
	0.1072*	70.3*	30.1
0.001	0.1088	71.1	
0.01	0.1091	70.8	
0.05	0.1092	70.9	
0.10	0.1090	70.7	30.9
0.30	0.1073	70.9	30.6
0.50	0.1057	71.7	30.4
1.0	0.1044	72.4	30.4
2.0	0.1034	73.3	30.4
3.0	0.1019	74.3	30.3
5.0	0.0950	75.0	28.6

\* Calculated by Bertrand's formula.

0.5 per cent of concentrated hydrochloric acid solution. The mixture is stirred vigorously for about 20 minutes, when the muscle fibers have become nearly white. The container is allowed to stand several hours, or overnight. The total volume of alcohol and fibers is measured in a graduated cylinder and as large an aliquot as is convenient is filtered off through a dry, soft fluted paper into another measuring cylinder.

*Fat Separation.*—This alcoholic filtrate is evaporated at a low

temperature on a water bath, water being added as the evaporation proceeds, until an aqueous residue of small volume remains. If necessary, more HCl is added, until the liquid is acid to Congo red, together with about 0.5 gm. of KCl. The warm liquid is then agitated with benzene, which is also poured around the walls of the dish. Several small pieces of high melting paraffin (the amount depending on the quantity of fat present) are added, and dissolve rapidly in the benzene. The dish is now warmed carefully, under a small jet of air. When this is properly done the paraffin collects in a cake at the sides of the dish. After the mixture has cooled, the acid solution is poured off through a soft filter. The paraffin is washed with 0.2 per cent HCl solution. Sometimes a protein-like precipitate forms, which is slow to filter. More KCl may then be added.

*Isoelectric Extraction.*—To the acid filtrate (evaporated, if necessary, to a convenient volume, which for our extractions is about 60 cc.) about 1 cc. of syrupy phosphoric acid is added, then strong KOH solution until a drop of the liquid on a spot plate gives, with a drop of dilute thymol blue solution, a color corresponding to pH 9 (approximately) as shown by a known buffer solution similarly treated.

The turbid liquid is then transferred to the body of an automatic liquid-liquid extractor and extracted with  $\frac{2}{3}$  its volume of chloroform containing 20 to 25 per cent alcohol. Into the bulb of the extractor—out of contact with the aqueous solution—5 or 6 drops of N HCl solution are introduced. Controls with the particular extractor used are necessary to determine the length of time needed for extraction. In our machines an hour is ample to remove completely 25 mg. of morphine. It is unwise to run the extractor much longer than necessary. Sometimes the extracted liquid foams, especially at first. As long as the foam is not carried over into the reservoir of solvent, it is of no moment, but if this appears likely to happen, the extractor may be run for a while at a lower speed. Frequently a few drops of soap solution, added to the aqueous layer, will stop the foaming; powdered talc also does this, but retains some morphine.

In the analysis of muscle tissue, the first extract with alcohol-chloroform still contains substances interfering with the subsequent estimations, but a second extraction removes them. Ac-

cordingly, the morphine-containing solvent is evaporated at low temperature, until nearly dry; acidulated water is then added, the solution buffered with phosphates as before, adjusted to pH 9, and then again extracted with alcohol-chloroform. The second extraction never presents any difficulties such as foaming.

The chloroform extract is cautiously evaporated, and the residue, when near to dryness, is taken up in 5 to 6 cc. of water containing a little HCl. The liquid is filtered into a small test-tube,

TABLE V.  
*Morphine Recoveries in Blank and Control Determinations on Normal Dog Muscles.*

Muscle taken.	Morphine taken.	Morphine found.	Ignition residue.	Recovery.
gm.	mg.	mg.	per cent	per cent
20	32.0	32.0	71.5	100
20	21.3	21.9	72.0	103
20	21.3	19.8	71.1	93
20	0	0		
20	21.3	19.6		92
20	0	0.7		
20	21.3	20.9	70.8	98
20	0	0.9		
20	21.3	22.0	71.7	105
10, 5 cc. blood.	32.0	32.2	71.5	101
20	30.3	30.5	71.5	101
20	30.3	30.0	71.3	99
20	30.3	30.2	70.2	100

The figures in groups represent consecutive determinations.

15 mg. of KCl added, and the silicotungstate precipitation carried out as described above. The amount of HCl used should make the solution, just before precipitation, between 0.05 and 0.1 N.

Data showing the recoveries obtained in blank and control determinations on normal dog muscles are given in Table V.

With muscle which was partially decomposed, this method gave, however, very unsatisfactory results, which were evidently much

too high. That these figures were unreliable was at once apparent because the ignited residue from the silicotungstic acid precipitate was several per cent higher than the value for the morphine salt. A blank determination showed that some other precipitable material was being isolated with the morphine, which gave an ignition residue of 86.5 per cent. On the assumption that the high results were due to the presence of a definite substance in the precipitate, it was, of course, possible to calculate the actual

TABLE VI.

*Recoveries of Morphine from Dog Muscle, Calculated from Precipitates, Showing an Incorrect Value of Residue on Ignition.*

Muscle taken.	Morphine taken.	Residue on ignition of silicotungstate precipitate.	Morphine recovered as calculated.	Recovery.
gm.	mg.	per cent	mg.	per cent
0	20	70.3		
20	8.4	73.7	7.0	86
20	16.8	76.9	18.0	106
20	32.0	77.8	35.0	109
20	32.0	76.2	28.0	88
20	32.0	74.3	32.0	100
20	21.3	75.5	21.0	98

amount of morphine when given the weight of the precipitate and that of the ignited oxides. A simple calculation gives the formula.

$$\text{Wt. of morphine silicotungstate} = \frac{(0.865 \times \text{wt. of dried silicotungstate}) - (\text{wt. of oxide residue})}{0.162}$$

This formula will be seen to exaggerate any error in determining the percentage of non-volatile substances in the precipitate. However, the results shown in Table VI are curiously close to the correct values.

In every case which we have seen of a control determination on dog muscle having an abnormally high non-volatile residue for the silicotungstic acid precipitate, the formula gave figures close to the correct result. This calculation is practically empirical and is not recommended as a method. On the contrary, such results should not occur with ordinary tissues. The matter is

mentioned, however, to illustrate the value of a check on the morphine determination, and perhaps, in preliminary experiments, to serve in an emergency.

Estimations of morphine in muscle may also be readily made by the benzyl alcohol extraction process described elsewhere (23).<sup>7</sup> This method is slower and much more expensive. For tissues containing quantities of lipoids, however, no alternative procedure has been found as satisfactory. It serves excellently for the analysis of brain, nerve tissue, fat, and the like.

*Method for Urine.*—The existing methods seemed to us particularly imperfect when applied to urine. The removal of urinary pigments and bases is very difficult, and nothing is gained by an original alcohol extraction, such as we employed with muscle. Morphine is not readily extracted at pH 9 by pure chloroform, but easily comes out in chloroform-alcohol, accompanied, however, by a large amount of pigment and other substances precipitable by silicotungstic acid.

It seemed as though the principle underlying Takayanagi's method might be used if a way of avoiding the evaporation of alkaline morphine solutions could be found. First, however, it became necessary to find out something about the relative extractibility of morphine and other precipitable substances at different pH levels. Accordingly, from a large sample of dog urine, 60 cc. portions were adjusted to different pH levels, with either  $H_3PO_4$  or KOH. They were all extracted in one of our continuous extractors for 2 hours, with 90 cc. of chloroform-alcohol (sp. gr. 1.308 at 22°). The extracts were evaporated, then precipitated with silicotungstic acid, and the weights of the precipitates plotted against the pH at which the extracts were obtained.

Similarly from water solutions of morphine and buffer salts at different pH levels, as much morphine as could be extracted in 2 hours was determined in the same way, and also plotted.

The curves in Fig. 1 show these data. It can be seen, there-

<sup>7</sup> We have, however, changed our opinion concerning the amyl alcohol extraction of morphine, which was stated to be as good as the chloroform-alcohol process. We now believe that high results are caused by use of amyl alcohol, particularly in the analysis of muscle.

fore, that at a pH of about 4 the extraction of interfering substances is rapid, while the removal of morphine is practically zero.

The method based upon this is therefore simple: 60 cc. of urine (if more is used, and evaporated, take great care that it is never strongly heated or alkaline) is adjusted to pH 9 by addition of KOH solution. This amount is then extracted with 90 cc. of chloroform-alcohol of sp. gr. 1.308 (for  $\frac{3}{4}$  hours in our apparatus), 5 to 6 drops of N HCl being placed in the receiver of the extractor. The liquid may foam, but this can be neglected. The extract, evaporated to remove chloroform, is diluted by water, then

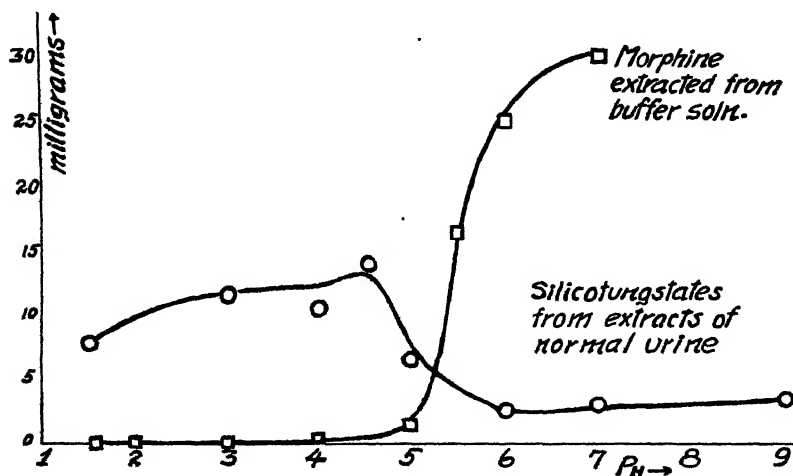


Fig. 1.

$\text{H}_3\text{PO}_4$  and KOH are added to produce a pH 3 (approximately). Extraction is again performed until the chloroform layer is colorless, after which the extract is discarded. The extracted liquid, now nearly colorless, is adjusted to pH 9.0 with KOH, the morphine removed by a third chloroform-alcohol extraction, and then determined in the extract precisely as directed previously.

By this method, coupled with a preliminary extraction with HCl-alcohol and a fat separation as with muscle, the morphine content of dog feces may be determined. With firm, friable feces, obtained from a bone ash diet, the results are satisfactory, but with diar-

reanal excreta, or feces containing much undigested food, the results are high and very variable. The determinations can only be accepted when the ignition residue of the precipitate gives the correct value. Typical results are given in Table VII.

*Method for Blood.*—20 to 25 cc. of blood are placed in a suitable flask and quickly shaken together with about half a liter of absolute alcohol containing 1 per cent acetic acid, and also 3 to 5 gm. of cetyl alcohol. The precipitate is broken up as much as possible and stirred for at least 30 minutes with an efficient motor stirrer.

TABLE VII.  
*Control Determinations on Normal Dog Urine and Feces.*

Material used.	Morphine added.	Morphine recovered.		Ignition residue.
	mg.	mg.	per cent	per cent
50 cc. water.....	28.8	26.7	93	
50 cc. urine.....	0	0	0	
50 " " .....	30.3	27.2	90	69.7
50 " " .....	30.3	26.3	87	70.7
50 " " .....	30.3	26.2	87	69.3
50 cc. urine.....	30.3	30.7	101	70.4
40 gm. feces (dry).....	0	1.3		
40 " " " .....	30.3	29.1	96	70.9
50 " " " .....	0	2.7		
50 " " " .....	30.3	26.8	89	71.4
50 " " " .....	0	1.0		
50 " " " .....	30.3	29.8	98	71.4

The figures in groups represent consecutive determinations.

The material, shaken occasionally, is allowed to stand for several hours or overnight, and a large aliquot of the liquid obtained by filtration, as described for muscle. After the filtrate is evaporated at a low temperature, a separation of the fat in a cake of paraffin is made, followed by a single extraction of the morphine at pH 9 by the alcohol-chloroform mixture. The extract is treated as described under muscle analysis.

As previously shown, there is a retention of morphine by the blood proteins coagulated in acetic acid-alcohol. The more



efficient hydrochloric acid-alcohol, which completely extracts morphine from muscle, cannot be employed, since it also dissolves a large amount of hemoglobin derivatives, which subsequently interfere greatly with the analysis. The amount of adsorbed morphine can, however, be reduced to a minimum by the use of cetyl alcohol and similarly acting substances.

TABLE VIII.  
*Control Experiments Showing Recovery from Liver and Blood.*

Material analyzed.	Morphine taken.	Morphine found.	Ignition residue.	Recovery.
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
13 gm. liver.....	16.8	16.8	72.3	100
18 " " .....	0	1.0		
60 gm. liver.....	75.7	55.4	70.4	73
60 " " .....	75.7	60.2	72.7	80
20 " " .....	30.3	24.7	71.2	81
20 " " .....	30.3	23.9	70.3	79
25 cc. blood.....	14.8	14.4	70.5	97.5
25 " " .....	14.8	15.0		101
25 " " .....	8.3	7.6		92
25 " " .....	16.5	15.4	71.0	93
25 " " .....	24.8	22.4	71.5	90
25 " " .....	16.5	14.9	70.9	90
25 " " .....	16.5	15.0	70.0	91
25 " " .....	33.2	31.2	71.2	94
25 " " .....	16.5	15.4	70.5	94
25 " " .....	8.3	6.9	70.5	84
25 " " .....	24.8	22.9	70.4	92
25 " " .....	0	0		
25 " " .....	0	0		

The figures in groups represent consecutive determinations.

This method for blood has given, in addition, satisfactory results with liver tissue (Table VIII), and with simple proteins such as egg albumin. Certain foreign substances, probably peptones, may seriously interfere with the manipulations in the test, generally impeding filtration. Heparin, used in a perfusion experiment, made filtration of the evaporated alcohol extracts almost impossible.

## SUMMARY.

Several sources of error in the existing methods of determining morphine have been pointed out. When the morphine is isolated by the precipitation of accompanying impurities, there is apt to be a retention of morphine by the precipitate, which in some cases cannot be removed by exhaustive washing. Methods which avoid the formation of precipitates from morphine solutions frequently require evaporation of neutral or alkaline morphine solutions. Such evaporation decomposes a considerable amount of morphine. The decomposition products may follow the morphine through the analysis, and may finally be estimated with it, because of the similarity in reactions between morphine and some of its oxidation products. This precludes any distinction, however, between morphine and its oxidation products in the original material. Control determinations on tissues containing known amounts of morphine may very well give satisfactory results, without indicating that the method is suitable for the estimation of unchanged morphine in the presence of oxidized morphine.

A series of methods for estimating morphine is proposed which it is hoped eliminates many of these errors. The methods are unsuitable for amounts of morphine less than 20 mg. per 100 gm. of material, but where applicable they possess the advantages of rapidity, simplicity, exclusion of the oxidation products of morphine, and inclusion of a desirable check on the final result.

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## THE OPTICAL ACTIVITY OF PSEUDOMORPHINE.\*

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During a study of some of the oxidation products of morphine it was necessary to prepare and identify pseudomorphine. As criteria of the success of the preparation, the known properties of the substance were observed. Among the characteristic properties of pseudomorphine, the optical rotation in alkaline solution is given (Hesse (1)). While the substance prepared by us according both to Denigès (2) and Vongerichten (3) corresponded in other details with pseudomorphine as described in the literature, we were unable to confirm the values recorded for its optical activity. This led to a closer scrutiny of the optical behavior.

Pseudomorphine is an alkaloid obtained by the gentle oxidation of morphine, and consists of two morphine molecules joined together with the loss of a hydrogen atom from each one. It has been shown (3) that the molecule contains intact the hydroxyl groups of the original morphine, and since one of these is phenolic, pseudomorphine, like morphine, dissolves in both acids and alkalis with salt formation. A slight excess of alkali over that corresponding to the monometallic salt is necessary for solution, on account of the hydrolysis of the phenolate. Since the free base is never pure, preparations of the hydrochloride, pseudomorphine  $\cdot$  2 HCl, were used, in solution containing an excess of potassium hydroxide. In view of the high concentrations of alkali used in these experiments, the hydrolysis has been considered negligible, and three molar equivalents of alkali have been calculated as removed from the solution to neutralize the hydrochloric acid and to form the potassium pseudomorphinate.

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The measurements were made at  $28^\circ \pm 1^\circ$ , usually on 2 dm. columns of liquid. A Schmidt and Haensch triple field polariscope was used, for which D light was supplied from an attached spectro-scope. The instrument could be read to  $0.01^\circ$  and the arithmetical mean of five or seven readings was taken, but our experience in matching the fields indicated that the observer's eyes could not be depended upon closer than  $0.02^\circ$  in these averages. Since this is quite the largest error in the measurements, the size of the

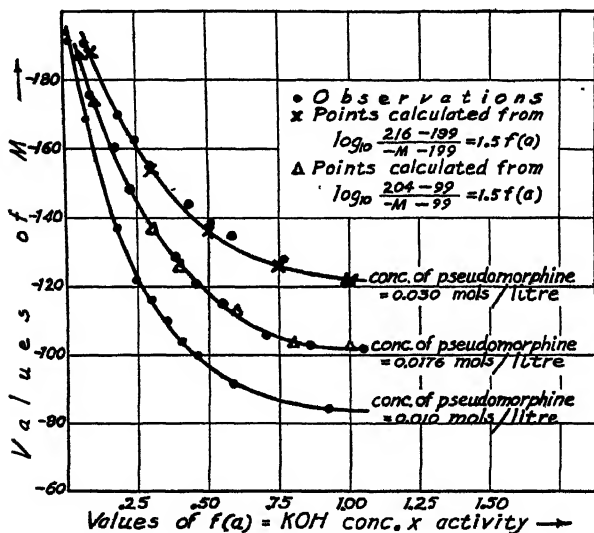


CHART I. Rotation of pseudomorphine solutions at constant pseudomorphine concentration and varying amounts of KOH.

original readings, shown in the data, is an indication of their probable accuracy.

It became evident that the higher the concentration of pseudomorphine the higher the observed rotation, but the higher the concentration of alkali, the lower the rotation. Under conditions for maximum rotation  $[\alpha]_D$  is about  $-370^\circ$ , showing pseudomorphine to be among the most optically active substances known. There seems to be no lower limit for dilute and strongly alkaline solutions, within the range of reliable observations.

Since the variation seemed to be due to two factors, one connected with the concentration of KOH and the other with the concentration of pseudomorphine, each of these variables was kept constant in a series of experiments, leading to the results shown in Charts I and II.

When pseudomorphine is constant in concentration, but the alkali is varied, a family of curves is produced, each member of which corresponds to a particular concentration of alkaloid. The reverse is true when the alkaloid is varied, each curve corresponding

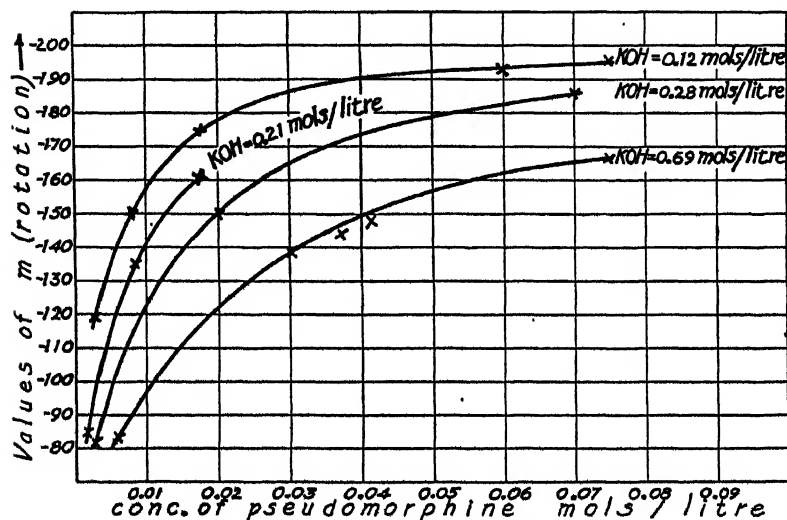


CHART II. Rotation of pseudomorphine solutions at constant alkali concentration and varying amounts of pseudomorphine.

to a particular concentration of KOH. It is possible to work out empirical equations to represent each curve separately, differing from each other only by numerical constants. They are simple exponential expressions, but of no particular use in correlating the data, since each equation represents a special case. The agreement between observed and calculated values is good and may be improved by multiplying the concentration of KOH ( $a$ ) by its activity coefficient ( $f$ ) and using the product instead of the actual concentration. Values for the activity coefficient of

KOH are taken from Harned (4). Since the density of these solutions is practically a function of their alkali content, it seemed more reasonable to express their rotation on a volume basis, and this has been done throughout by omitting the usual density factor included in the specific rotation of solutions.

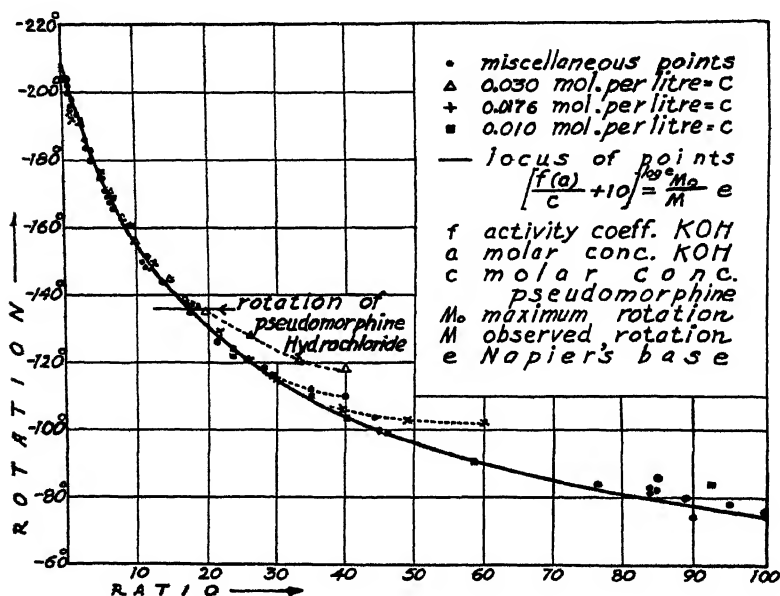


CHART III. Rotation of pseudomorphine solutions, plotted against ratio  $\frac{\text{mols KOH} \times \text{activity coefficient}}{\text{mols pseudomorphine}}$ .

For simplicity, the data have been calculated to a molar basis, where  $M$ , the number used here to express rotation, is defined by the expression  $M = [\alpha]_D^{25} \times 0.568$ .<sup>1</sup>

The relationships shown by these curves may be combined by plotting rotation against the ratio of alkali concentration to pseudomorphine concentration, which brings all of these isolated observations into a simple relationship (Chart III).

<sup>1</sup> The molar weight of pseudomorphine is taken as 568.  $M$  is therefore the angular rotation corresponding to a 1 dm. column, divided by the molar concentration of pseudomorphine, and without regard for the density of the solution.

Except for certain solutions which we have some reason to regard as supersaturated, the optical rotation of pseudomorphine depends neither on the concentration of pseudomorphine nor alkali, but upon the ratio of the two. In other words, *for a given ratio of pseudomorphine and alkali the same rotation will be observed, irrespective of the actual concentrations.* As the proportion of alkali to pseudomorphine increases, the rotation decreases, following a single well defined curve, which is the locus of practically every point of the several score which we had previously determined.

The points on the dotted curves may represent supersaturated solutions, because many of them deposit a precipitate if they are shaken or kept a few days.

On the assumption, entirely without *a priori* justification, that this curve approaches zero, an empirical equation may be set up,

$$M \left[ \frac{f(a)}{c} + p \right]^q = K$$

in which  $p$ ,  $q$ , and  $K$  are constants,  $f$  is the activity coefficient of the KOH,  $(a)$  = molar concentration of KOH,  $c$  = molar concentration of pseudomorphine, and  $M$  = the rotation of the solution calculated as described above. The values of the constants were obtained from a smoothed curve, drawn through the observation points. Numerically their values are quite curious:  $q = 0.435$ ,  $p = 10$  (nearly), and  $K = -572$  (nearly).

The maximum rotation,  $M_0$ , (unattainable in practice on account of hydrolysis) may be calculated from the preceding formula to be  $-210$ . It is interesting, if inexplicable, that  $M_0 \times$  the natural constant  $e = K$  very nearly, and this purely empirical formula may be written

$$\left[ \frac{f(a)}{c} + 10 \right]^{\log_{10} e} = \frac{M_0}{M} e$$

The agreement between this formula and the observed values is extraordinarily close over the entire range of our observations (Table I).

Chemically, it is difficult to see what may be happening in these solutions. The various possibilities of the dissociation theory are apparently all contradicted by some of the observed



TABLE I.

*Rotation of Pseudomorphine in KOH Solutions.*

Symbols as in text.

$\alpha$	$c$	$\frac{f\alpha}{c}$	$-M$ calculated.	Observed readings in degrees of arc, corresponding to a 1 dm. column.	$-M$ observed.
<i>mols per l.</i>	<i>mols per l.</i>		<i>degrees</i>		<i>degrees</i>
0.0529	0.0882	0.49	204	18.00	204
0.0705	0.0705	0.79	202	14.25	202
0.0152	0.0397	0.39	205	7.94	200
0.0529	0.0360	1.21	197	7.12	198
0.112	0.0745	1.2	197	14.60	196
0.0176	0.0176	0.95	198	3.41	194
0.112	0.0596	1.5	194	11.50	193
0.028	0.0176	1.36	196	3.28	192
0.197	0.075	2.00	191	14.24	190
0.085	0.0300	2.5	190	5.73	191
0.271	0.0705	2.86	187	13.10	186
0.253	0.0528	3.6	182	9.66	183
0.126	0.0318	3.1	186	5.85	184
0.165	0.0372	3.5	183	6.71	181
0.118	0.0176	5.22	175	3.10	176
0.235	0.0353	5.0	176	6.18	175
0.344	0.0441	5.8	171	7.55	171
0.241	0.0300	6.0	170	5.10	170
0.120	0.0159	5.9	171	2.68	169
0.086	0.0100	6.9	167	1.69	169
0.687	0.0750	6.7	168	12.46	166
0.542	0.0617	6.4	169	10.37	168
0.319	0.0300	7.9	162	4.89	163
0.397	0.0300	9.8	156	4.68	156
0.218	0.0176	9.5	157	2.83	161
0.117	0.0079	11.5	150	1.19	150
0.298	0.0176	12.6	147	2.62	149
0.145	0.0100	11.2	152	1.48	148
0.685	0.0420	11.9	149	6.19	147
0.585	0.0300	14.3	143	4.34	145
0.685	0.0372	13.5	144	5.37	144
0.687	0.0300	16.8	136	4.16	139
0.242	0.0100	18.1	134	1.37	137
0.418	0.0176	17.4	135	2.41	137
0.209	0.0088	18.1	134	1.19	135
0.787	0.0300	19.5	130	4.08	136

TABLE I.—*Concluded.*

<i>a</i>	<i>c</i>	$\frac{fa}{c}$	<i>-M</i> calculated.	Observed readings in degrees of arc, corresponding to a 1 dm. column.	<i>-M</i> observed.
<i>mols per l.</i>	<i>mols per l.</i>		<i>degrees</i>		<i>degrees</i>
0.626	0.0264	17.7	135	3.56	135
0.518	0.0176	21.4	128	2.27	129
1.01	0.0300	25.7	120	3.87	129
0.58	0.0199	21.2	128	2.51	126
0.320	0.0100	23.7	124	1.22	122
1.24	0.0300	32.8	111	3.66	122
0.618	0.0176	25.7	121	2.13	121
1.47	0.0300	40.0	104	3.59	119
0.115	0.0032	28.0	118	0.38	119
0.398	0.0100	29.3	116	1.16	116
0.718	0.0176	29.9	115	2.04	116
0.204	0.0044	35.2	110	0.59	112
0.477	0.0100	34.8	109	1.10	110
0.918	0.0176	39.6	104	1.88	107
1.118	0.0176	49.2	97	1.83	104
1.318	0.0176	59.8	90	1.81	103
0.554	0.0100	40.3	104	1.04	104
0.421	0.0071	44.4	100	7.33	104
1.95	0.0199	87.0	78	2.05	103
0.631	0.0100	46.1	99	1.00	100
0.788	0.0100	58.4	91	0.92	92
3.90	0.0100	420.0	41	0.91	91
0.202	0.0018	85.0	79	0.15(5)*	86
1.00	0.0100	76.0	82	0.84	84
1.176	0.0100	91.8	77	0.85	85
1.95	0.0100	175.0	59	0.85	85
0.692	0.0060	84.0	80	0.49(5)	83
0.283	0.0025	85.0	79	0.20(5)	82
0.360	0.0030	89.0	78	0.24(0)	80
0.321	0.0025	95.0	75	0.19(5)	78
0.338	0.0025	100.0	74	0.19(0)	76
0.300	0.0025	90.0	76	0.18(5)	74
2.44	0.0008	2760.0	18	0.05(5)	69
0.709	0.0036	144.0	64	0.24(5)	63
2.50	0.0018	1365.0	25	0.10(8)	60
2.44	0.0004	5750.0	13	0.02(1)	52

\* When the readings were low, twelve or more observations were made and averaged. The figure in parenthesis is the third decimal place of this average.

facts. When supersaturated with pseudomorphine these solutions, if heated, deposit crystals which analyses of the supernatant liquid indicate to be the monopotassium salt of pseudomorphine. One would conclude from this that no reaction has taken place during the addition of the extra alkali, which is in agreement with the conclusions of Vongerichten (3), regarding the modification of acidic properties in one phenol group of pseudomorphine. If kept in the cold, these solutions form a jelly of indeterminable composition, which, however, withdraws large quantities of water from the solution. This is typical of colloids, and it is possible

TABLE II.

*Effect of KCl on the Rotation of Alkaline Pseudomorphine Solutions.*

Symbols as in text.

$\alpha$	$c$	Additional KCl.*	Observed $M$ .	$M$ calculated.
		<i>mols per l.</i>	<i>degrees</i>	<i>degrees</i>
0.360	0.003	0	80	78
0.360	0.003	0.006	78	78
0.360	0.003	0.018	80	78
0.117	0.008	0	151	150
0.117	0.008	0.081	153	150
0.117	0.008	0.210	154.5	150
0.117	0.008	1.21	170	150
0.117	0.008	2.62	173	150
0.117	0.0037	3.36	184	150

\* Due to the use of the hydrochloride of pseudomorphine, all the solutions contained 2 mols of KCl per mol of pseudomorphine.

the explanation of this behavior belongs to colloid rather than to molecular chemistry.

While we cannot venture for a moment upon the theoretical explanation of this property of pseudomorphine, we have without success searched both the literature and the laboratory for substances whose optical behavior is similar. As a means of identifying this substance, its optical rotation appears to offer a reliability exceeded by few tests.

Charts I and II show no resemblance to dissociation curves, while Chart III might be regarded as roughly doing so. However,

since the  $x$  axis measures a ratio, which is quite independent of the actual concentrations in the system, any interpretation of the curve based upon different rotations for dissociated and undissociated pseudomorphine, or for mono- and (hypothetical) dipotassium pseudomorphinate, or their dissociation products, does not appear to fit the case.

The hydroxyl ion, nevertheless, seems to be responsible for the decrease in optical activity, since if the K ion concentration is raised by the addition of KCl, no effect upon the rotation is observed with small amounts, but with large amounts of KCl, the rotation is somewhat increased (Table II). This is hardly due to the lowering effect of KCl upon the activity of the KOH, since this occurs most markedly with small amounts of chloride (4).

When the concentration of KOH is itself greatly increased beyond that recorded in our measurements, a precipitate of monopotassium pseudomorphinate usually forms. If, however, a relatively small concentration of pseudomorphine is employed, (0.01 M) considerable alkali may be added without apparently saturating the solution. In such cases, the rotatory power is also greatly increased. Such an effect may be the cause of the deviations marked by dotted lines in Chart III. It is interesting that these deviations begin (as nearly as can be measured) at approximately the same alkali concentration in each case (about 0.6 M).

It appears then that the hydroxyl ion, by some specific action, reduces the rotatory power of pseudomorphine,<sup>2</sup> while increasing the concentration of dissolved substances by adding neutral K salts, pseudomorphine, or even KOH itself, tends toward saturation of the solution with respect to K pseudomorphinate and an increase in the optical activity.

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<sup>2</sup> This action of  $\text{OH}^-$  upon optically active substances has been frequently observed, but is not of sufficient amount to allow as accurate measurement as in the present case. The specific rotations of many sugars and amino acids are numerically lowered by additions of alkali.



## THE EFFECTS OF PROTEIN SPLIT-PRODUCTS UPON METABOLISM.

### III. FURTHER INVESTIGATION OF THE FRACTIONATED PROTEIN HYDROLYSATES AND OF AMINO ACIDS, AND THEIR RELATION TO THE SPECIFIC DYNAMIC ACTION OF THE PROTEINS.

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This communication represents a continuation of the attempt to throw light upon the specific dynamic action of proteins through a study of the split-products of these foodstuffs. Observations had been made (Rapport, 1926-27), indicating that simple hydrolysis of the proteins did not appreciably alter their effect on metabolism. Later experiments (Rapport and Beard, 1927) showed that upon fractionating the casein and gelatin hydrolysates by the butyl alcohol extraction method of Dakin (1920), the specific dynamic action of the first fraction, containing the monoaminomonocarboxylic acids could be accounted for by the theoretical summated effects of its constituent amino acids. It was incidentally observed that phenylalanine and tyrosine had conspicuous effects upon the heat production, the former considerably greater than that of glycine, an observation that has recently been confirmed by Wilhelmj and Bollman (1928).

But the acids of this series did not suffice to account fully for the specific dynamic action of proteins. Consequently one of two alternatives followed. Either other amino acids would be found in the remaining fractions to have a hitherto unestablished effect, upon ingestion, in increasing the metabolism; or the formation of compounds in the nature possibly of polypeptides must be postulated to occur during the course of protein catabolism, to which

that portion of the specific dynamic action of the proteins which was still unaccounted for could be ascribed.

It was our purpose to make systematic inquiry into the effects of the other fractions, namely the dicarboxylic acid fraction, the diamino acid fraction, and the fraction containing proline, hydroxyproline, and certain diketopiperazines, with a view to determine which of the alternatives mentioned above appeared more probable.

We have arrived at the conclusion that the summated effects of individual amino acids are responsible for the observed effects of the proteins, and that these amino acids include not only phenylalanine, glycine, tyrosine, alanine, and leucine, which had been previously shown to have a specific dynamic action, but also glutamic acid, aspartic acid, arginine, and cystine.

#### *Method.*

We would have preferred to carry out the present observations on the same animal that was used by us in the previous work dealing with this general subject. Unfortunately the dog died, and we were constrained to use another animal which had been employed before in certain experiments on muscular exercise. This dog (Dog 8) proved eminently satisfactory. It was given a daily diet of 75 gm. of lean beef heart, 75 gm. of lard, 15 gm. of cracker meal, and 5 gm. of white bone ash in addition to the experimental diets. The latter included two proteins—casein and gelatin—the dicarboxylic acid and diamino acid fractions of their hydrolysates, and certain amino acids. These substances were as a rule not sufficiently tempting to be taken voluntarily, and we found that the best method of administration was one we have adopted before; namely to make a paste of the material in question with as little water as possible, and to place it on the back of the tongue, when it was perforce swallowed.

The hydrolysis of the protein and extraction of the monoamino-monocarboxylic acids by means of butyl alcohol was given in Paper I of this series (Rapport and Beard, 1927). The fractions used in this research were prepared by the method of Dakin (1920) as follows.

*Proline Fraction.*—The butyl alcohol solutions were evaporated under reduced pressure and the residue was taken up in ethyl alcohol and allowed to stand several days at 0° to separate traces

of other acids. A slight precipitate was obtained. The alcoholic filtrates were boiled out many times with norit, but it was practically impossible to remove all the black pigment from these proline residues. The alcohol was then evaporated off and residues were kept in the cold room until used.

*Diamino Acid Fraction.*—The hydrolysates from both gelatin and casein were extracted with butyl alcohol until no more precipitate of monoamino acids appeared in the extraction flask. They were then separately evaporated to about three-fourths the original volume to remove butyl alcohol. The diamino acids were completely precipitated by the procedure outlined by Vickery and Leavenworth (1928), by means of 20 per cent phosphotungstic acid in 5 per cent sulfuric acid solution. The phosphotungstates were thoroughly washed with 2 per cent phosphotungstic acid in 5 per cent sulfuric acid solution. The filtrate was reserved for the preparation of the dicarboxylic acids below.

The bulky precipitate of phosphotungstates was decomposed by suspension in 80 per cent acetone, dilution with water, and then addition of solid calcium hydroxide. After neutralization a large excess of calcium hydroxide was added to precipitate completely any phosphotungstic acid that might have remained in solution. The precipitates of calcium sulfate and phosphotungstate were filtered off, washed with lime water, and the filtrate and washings were almost neutralized with sulfuric acid. A small precipitate of calcium sulfate was filtered off and washed. Ammonia was removed by evaporating the slightly alkaline solution *in vacuo* to dryness.

*Dicarboxylic Acid Fraction.*—Sulfuric and phosphotungstic acids were removed from the filtrate obtained above from the phosphotungstates of the bases and made strongly alkaline with calcium hydroxide. The solution was filtered and concentrated on the steam bath to a small volume. It was added from a dropping funnel to a large volume of alcohol (150 cc. of solution to 1500 cc. of alcohol), which was continually stirred. The precipitated salts, containing the aspartic, glutamic, and other dicarboxylic acids if present, together with some glycine, were washed with alcohol, dissolved in water, and the calcium removed exactly with sulfuric acid. The solution was concentrated and allowed to stand in the cold room at  $-10^{\circ}$  for a few days for the acids to crystallize.



*Removal of Glycine.*—Since some glycine may be present in the above fraction it was removed as follows: 100 gm. of the dicarboxylic acid fraction of gelatin were dissolved in hot water and saturated twice with dry gaseous hydrogen chloride. The resulting glutamic acid hydrochloride was removed in two crops, treated with the calculated amount of sodium hydroxide to combine with the hydrochloric acid, and the solution evaporated to dryness.

The filtrate from the glutamic acid hydrochloride was evaporated to dryness, dissolved in absolute alcohol, and saturated three times with dry gaseous hydrogen chloride. A very small amount of glycine ester hydrochloride was obtained. The glutamic acid residue was suspended in the glycine-free filtrate, thoroughly mixed, and the whole evaporated to dryness.

The metabolism was obtained over a period of usually 4 hours, beginning 1 hour after the ingestion of the substance to be studied, and at least 18 hours after other food. The apparatus used was a closed system of the Benedict-Homans type, and the  $\text{CO}_2$  and  $\text{O}_2$  exchange, together with the nitrogen excreted during the experimental period, permitted calculation of the heat production in the customary manner. As in former work, we were confronted by an obvious difficulty in connection with the nitrogen excretion. To calculate the heat production on the ordinary factors, which assume that the nitrogen excretion represents the catabolism of meat protein, is naturally unsound theoretically when, for example, a mixture of dicarboxylic acids is ingested. The conditions, to which we have previously called attention (Rapport and Beard, 1927), are not simple, and it would be bootless and time-consuming to discuss them in detail now. When, as in the case of glutamic acid and aspartic acid, the combustion heat of the substance was known, the factors for this substance were employed. At other times we used the conventional factors. It suffices to say that our calculations have shown the error to be negligible in a consideration of the total metabolism.

#### *Alcohol Checks.*

In six alcohol checks performed during the course of the work, covering a period of 2 months, the maximum deviations from the theoretical R.Q. of 0.667 were one of 0.673 and one of 0.659, indicating maximum errors of +0.9 and -1.2 per cent. The mean error was 0.2 per cent.

## EXPERIMENTAL.

## 1. Basal Metabolism.

During the course of 2 months, ten determinations of the basal metabolism were made. The average heat production per hour was 11.4 calories (Table I); the average  $O_2$  consumption 3.39 gm. The maximum deviations from the average heat production were + 2.1 and - 2.3 per cent; from the average  $O_2$  consumption +2.1 and -3.0 per cent. In determining the specific dynamic action of

TABLE I.  
*Basal Metabolism.*

Date.		Urinary N per hr.	R.Q.	$O_2$ per hr.	Calories per hr.
1928		gm.		gm.	
Jan.	5.....	0.103	0.88	3.46	11.65
"	7.....	0.095	0.83	3.34	11.14
"	10.....	0.103	0.85	3.45	11.53
"	15.....	0.100	0.84	3.39	11.29
"	18.....	0.101	0.90	3.43	11.62
"	21.....	0.084	0.85	3.38	11.32
"	25.....	0.101	0.88	3.41	11.52
"	30.....	0.093	0.92	3.31	11.27
Mar.	1.....	0.080	0.94	3.40	11.67
"	8.....	0.092	0.95	3.29	11.29
Average.....		0.096	0.88	3.39	11.43

Maximum deviation of  $O_2$  consumption = +2.1 and - 3.0 per cent.

" " " heat production = +2.1 " - 2.5 " "

a substance, we have deemed it advisable to speak in terms of the increase in the  $O_2$  consumption rather than in the heat production. This is probably more nearly accurate, though the difference is hardly appreciable.

Incidentally, although the dog's weight increased from 8.0 to 8.8 kilos during the 2 month period (a 10 per cent change) there was no corresponding change in the basal heat production. It seems clear that the deposited tissue had no appreciable metabolism. Yet with any of the formulæ involving weight, a 10 per cent reduction in the metabolism per sq. m. of surface area would be deduced, and one might suppose that the energy exchanges in

the "active protoplasmic mass" had been similarly decreased. There is no reason to believe that such was the case, and the error would seem in such a circumstance to lie in the use of the formula.

### 2. The Dicarboxylic Acid Fraction.

On three occasions 15 gm. of the dicarboxylic acid fraction of the gelatin hydrolysate, containing glutamic and aspartic acids (the contaminating glycine having been removed), were administered (Table II), resulting in an increased metabolism of 16, 10,

TABLE II.

*Experiments with Casein and Gelatin Dicarboxylic Acid Fraction.*

Average per hour of 4 hour period, unless otherwise noted.

	Date.	Urinary N.	O <sub>2</sub>	R. Q.	Calories.	Increase over basal O <sub>2</sub> .	Remarks.
	1928	gm.	gm.			per cent	
Dicarboxylic acid fraction (casein) 15 gm.	Jan. 6	0.143	3.95	0.87	13.22	16.5	3 hr. period. Animal restless during 4th hr.
	" 9	0.139	3.72	0.89	12.54	9.7	
	" 11	0.126	3.90	0.87	13.12	15.0	
Average.....						13.7	
Dicarboxylic acid fraction (gelatin) 15 gm.	Jan. 12	0.156	3.87	0.90	13.01	14.2	

and 15 per cent respectively, an average rise of 14 per cent. An experiment with the similar fraction of casein (containing hydroxy-glutamic acid in addition to the acids found in gelatin) caused the O<sub>2</sub> consumption to rise 14 per cent. It was evident that this fraction required further study. Grafe (1915) found that on giving a man 50 gm. of glutamic acid there was an increased metabolism of about 16 per cent; and that after the ingestion of 67.5 gm. of asparagine there was a heightened heat production in one instance but not in another. On the other hand Lusk (1912-13) was unable to find that glutamic acid had a specific dynamic

action, and Atkinson and Lusk (1918), after challenging the validity of Grafe's results with asparagine, found 10 gm. of

TABLE III.  
*Experiments with Glutamic Acid.*

	Date.	Urinary N.	O <sub>2</sub>	R. Q.	Calories.	Increase over basal O <sub>2</sub> .	Remarks.
	1928	gm.	gm.			per cent	
Glutamic acid 10 gm. at 9.15 a.m.	Jan. 20		3.77 3.78 4.05 3.97	0.91 0.93 0.86 0.87	12.64 12.71 13.41 13.12		Calories calculated on glutamic acid factors, assuming N above basal N as due to glutamic acid. Experimental period, 10.16 a. m.-2.16 p.m.
Average.		0.134	3.89	0.89	12.98	14.7	
Glutamic acid 10 gm. at 9.42 a.m.	Jan. 23		3.63 3.73 3.80 3.91	0.94 0.95 0.92 0.91	12.19 12.59 12.71 13.08		Experimental period, 10.45 a.m.-2.45 p.m.
Average.		0.140	3.77	0.93	12.65	11.2	
Glutamic acid 10 gm. at 9.25 a.m.	Jan. 31		3.68 3.56 3.85 3.93	0.96 0.99 0.97 0.96	12.51 12.09 13.11 13.39		Experimental period, 10.26 a.m.-2.26 p.m.
Average.		0.123	3.76	0.97	12.79	10.9	
Average of three experiments.....						12.3	
Glutamic acid 20 gm. at 8.45 a.m.	Mar. 6		3.62 3.84 3.73 3.88	0.99 0.93 0.97 0.89	12.27 13.16 12.65 12.93		Experimental period, 9.46 a.m.-1.46 p.m.
Average.		0.129	3.77	0.94	12.75	11.2	

aspartic acid to have no effect upon the total metabolism of an 11 kilo dog, and 15 gm. of asparagine to be without influence on

the metabolism of a 15 kilo dog.\* On the basis of his results Grafe concluded that the specific dynamic action of protein is largely due to liberation of free amino groups. We have had

TABLE IV.  
*Experiments with Aspartic Acid.*

	Date.	Urinary N.	O <sub>2</sub>	R. Q.	Calories.	Increase over basal O <sub>2</sub> .	Remarks.
	1928	gm.	gm.			per cent	
Aspartic acid 10 gm. at 9.15 a.m.	Jan. 24		3.68 3.64 3.67	0.93 1.00 0.98	12.48 12.45 12.55		3 hr. experiment. Animal restless during 4th hr. Heat calculated on aspartic acid factors, assuming N above basal N as due to aspartic acid. Experimental period, 10.16 a.m.-1.16 p.m.
Average.		0.128	3.66	0.97	12.52	8.0	
Aspartic acid 10 gm. at 9.23 a.m.	Feb. 2		3.52 3.59 3.71	0.94 0.96 0.98	11.99 12.28 12.71		3 hr. experiment. Animal restless during 4th hour. Experimental period 10.24 a.m.-1.24 p.m.
Average.		0.124	3.61	0.96	12.35	6.5	
Average of two experiments.....						7.3	
Aspartic acid 20 gm. at 8.45 a.m.	Mar. 7		3.55 3.70 3.72 3.78	0.98 0.97 0.96 1.00	12.11 12.64 12.68 12.92		Experimental period, 9.45 a.m.-1.45 p.m.
Average.		0.139	3.69	0.98	12.60	8.9	

occasion elsewhere (1927) to point out that this view is fallacious, but we agree with Grafe that glutamic and aspartic acids are capable when ingested of increasing the metabolism.

(a) *Glutamic Acid.*—In three experiments we gave to our dog

10 gm. of glutamic acid and found on each occasion a definite increase in the  $O_2$  consumption (Table III), the rises being, respectively, 15, 11, and 11 per cent, an average of 12 per cent. On another occasion we administered 20 gm. of the amino acid, but found that the specific dynamic action was not greater over a 4 hour period than on giving half that quantity, the increase being 11.2 per cent. This is probably due to slowness of absorption, so that the metabolizable units in the blood at any given time were no greater when the larger amount was administered than following the smaller, a conception which tends to be confirmed by the

TABLE V.

*Experiments with Casein and Gelatin Diamino Acid Fraction.*

Average per hour of 4 hour period, unless otherwise noted.

	Date.	Urinary N.	$O_2$	B.Q.	Calories.	Increase over basal $O_2$ .	Remarks.
	1928	gm.	gm.			per cent	
Diamino acid fraction (casein) 15 gm.	Jan. 16	0.121	3.68	0.89	12.46	8.6	3 hr. period. Animal restless during 4th hour.
	" 17	0.152	3.76	0.89	12.65	10.9	
Average.....						9.8	
Diamino acid fraction (gelatin) 15 gm.	Jan. 14	0.118	3.72	0.89	12.53	9.7	

nitrogen excretion, which was equally and only slightly elevated above the basal level in both instances.

(b) *Aspartic Acid*.—On administering 10 gm. of aspartic acid, we observed on two occasions a small but definite increase in the  $O_2$  consumption, amounting to 8 and 7 per cent, respectively (Table IV). In both of these experiments, the dog was restless during the 4th hour, and these results had to be discarded, but during each of the other 3 hours the small but unmistakable rise in the metabolism was evident. This rise was not increased by doubling the quantity of aspartic acid ingested (Table IV),

amounting to 9 per cent over the basal level, and we consider the explanation to be the same as in the case of glutamic acid.

The effect of the dicarboxylic acid fraction on the total metabolism, therefore, can be accounted for by the specific dynamic action of its constituent amino acids. Whether hydroxyglutamic acid contributes at all to the effect of this fraction of the casein hydrolysate, we are unable to say, as we had none of this amino acid to test.

### 3. The Diamino Acid Fraction.

The fraction contains arginine, histidine, and lysine. On two occasions 15 gm. of this fraction of the casein hydrolysate were administered, resulting in an increased  $O_2$  consumption of 9 and 11

TABLE VI.  
*Effect of Administration of 5 Gm. of Histidine Dichloride.*

Date.	Urinary N.	$O_2$	R.Q.	Calories.*	Remarks.
1928	gm.	gm.			
Mar. 2		3.38	0.97	11.50	2 hr. experiment. Histidine dichloride given at 9.20 a.m. Some movement during each of last 2 hrs.
		3.44	0.90	11.72	
Average.	0.130	3.41	0.94	11.61	

\* Calculated on ordinary protein factors.

per cent, respectively (Table V). In another experiment, the ingestion of 15 gm. of this fraction of the gelatin hydrolysate caused the  $O_2$  consumption to increase 10 per cent. We were therefore moved to investigate the individual acids of the fraction.

(a) *Histidine Dichloride*.—In a single experiment 5 gm. of histidine dichloride were given by mouth at 9.20 a.m. (Table VI). From 10.20 to 12.20 the gaseous exchange was obtained and there was absolutely no deviation of the metabolism from the basal level. The animal was kept in the chamber for 2 hours longer, but in each of these hours was somewhat restless so that the periods had to be discarded. We can only say therefore that histidine shows no specific dynamic action for at least 3 hours after its ingestion. The urinary nitrogen over a 5 hour period indicated an absorption of the amino acid comparable to that of glutamic acid.

(b) *Arginine Carbonate*.—When 5 gm. of arginine carbonate were given, however, there was a small but distinct rise in O<sub>2</sub> consumption above the basal level (Table VII), amounting to 6 per cent over a 4 hour period, and present during each hour, though tending to diminish in the later hours. We feel justified in saying that arginine has a specific dynamic action, which probably accounts for most of the effect of the diamino acid fraction.

We regret that the expense of the material has for the present prevented an examination of the effect of lysine.

#### 4. The "Proline" Fraction.

The fraction is extracted by, and soluble in, butyl alcohol. The method for obtaining it has already been described. We tried on

TABLE VII.  
*Effect of Administration of 5 Gm. of d-Arginine Carbonate.*

Date.	Urinary N.	O <sub>2</sub>	R.Q.	Calories.*	Increase over basal O <sub>2</sub> .	Remarks.
1928	gm.	gm.			per cent	
Mar. 5		3.63	0.90	12.25		Arginine carbonate given at 9.00 a.m. Experimental period. 10.02 a.m.-2.02 p.m.
		3.62	0.92	12.28		
		3.49	0.89	11.76		
		3.56	0.89	11.97		
Average.	0.134	3.58	0.90	12.08	5.7	

\* Calculated on ordinary protein factors.

various occasions to give this fraction of both the casein and the gelatin hydrolysates but without success, for it was invariably vomited, not only by this animal but by others as well, sometimes within a very short time, sometimes as late as 2 hours after its ingestion. We were hence obliged to abandon the effort.

#### 5. Effects of Casein and Gelatin Compared with Summated Effects of Fractions of Hydrolysates of These Proteins.

In Table VIII we have set down a comparison of the effect of giving casein or gelatin with the theoretical summated effects of the contained fractions. (In the case of the monoaminomonocarboxylic acid fraction the figures are taken from a report of



previous experiments (Rapport and Beard, 1927.) Thus in a quantity of casein containing 6 gm. of N, there were 12.96 gm. of the monoaminomonocarboxylic acid fraction. Theoretically this would have a specific dynamic action of about 15 per cent (Table VIII). Similarly, the contained dicarboxylic acid fraction would raise the  $O_2$  consumption about 15 per cent; and the amount of the diamino acid fraction in this amount of the protein would increase the metabolism about 4 per cent. The summated action of all three fractions (which accounts for the protein molecule except

TABLE VIII.  
*Effect of Gelatin and Casein Compared with Summated Effect of Fractions of Hydrolysates of These Proteins.*

Fraction.	Casein (44.6 gm.— 6 gm. N).			Gelatin (37.9 gm.— 6 gm. N).		
	Fraction in mole- cule.	Fraction in this amount of pro- tein.	Theoretical effect (increase of metabolism).	Fraction in mole- cule.	Fraction in this amount of pro- tein.	Theoretical effect (increase of metabolism).
	per cent	gm.	per cent	per cent	gm.	per cent
I. Monoaminomonocarboxylic acids.....	29.04	12.96	14.9	35.6	13.50	14.6
II. Dicarboxylic acids.....	36.37	16.40	14.7	9.3	3.53	3.2
III. Diamino acids.....	13.93	6.22	3.8	15.0	5.69	3.6
Glycine in aqueous residue.....				21.7	8.2	24.6
Total.....			33.4			46.0
Found on giving this amount of protein. ....			33.3			38.5

for the "proline" fraction) would therefore be about 34 per cent. When this quantity of casein itself was given, the increase in heat production was 33 per cent. In other words, the specific dynamic action of the protein is accounted for. In the case of gelatin the correspondence is not so exact, being 46 per cent as compared with 39 per cent, but is nevertheless close enough to validate our argument, to which we shall presently return. (In the case of gelatin, most of the contained glycine is not present in any of the fractions, and its theoretical effect must be added, as we have done in Table VIII.)

6. *Effect of Cystine.*

In an earlier experiment (Rapport, 1924) 1 gm. of cystine was given together with gelatin, and did not increase the specific dynamic action of the latter. Several possibilities existed to account for this: (1) the gelatin might have masked the effect of the cystine; (2) cystine might have no specific dynamic action; (3) the quantity of cystine given might not have produced a sufficient effect to be measurable. The influence of cystine on the specific dynamic action of the protein molecule must of course be negligible, in view of the fact that it is present only in very small percentage in nearly all of the common proteins. In casein and gelatin, for example, it exists, if at all, in minute traces. It is of

TABLE IX.  
*Effect of Administration of 10 Gm. of Cystine.*

Date.	Urinary N.	O <sub>2</sub>	r.q.	Calories.*	Increase over basal O <sub>2</sub> .	Remarks.
1928	gm.	gm.			per cent	
Mar. 9		3.60	0.87	12.45		Cystine given at 9.00 a.m. Experimental period, 10.02 a.m.-2.02 p.m.
		3.68	0.85	12.69		
		3.75	0.89	12.76		
		3.70	0.83	12.73		
Average.	0.120	3.68	0.86	12.66	8.6	

\* Calculated on cystine factors, N above basal N assumed to be due to cystine.

peculiar interest and importance, however, because of its relation to glutathione.

We have given 10 gm. of cystine to our dog, with a resultant increase of metabolism of 9 per cent (Table IX), the increase being present in each of the experimental hours. It appears then that cystine has a definite specific dynamic action, even though its contribution to the effect of the common proteins be inappreciable.

7. *Relative Specific Dynamic Action of Amino Acids.*

We have compiled in Table X the approximate effect, upon the total metabolism of 8 to 10 kilo dogs, of those amino acids which have been studied in this and previous researches. If the specific

dynamic action of glycine is arbitrarily taken as 100, it will be seen that, per gm. ingested, its effect is exceeded by that of phenylalanine, and is almost approached by that of tyrosine and alanine. In a definitely lower category lie leucine, cystine, glutamic acid, aspartic acid, and arginine. The effects of proline, hydroxyproline, serine, tryptophane, hydroxyglutamic acid, and lysine remain

TABLE X.  
*Specific Dynamic Action of Amino Acids (for Dogs of 8 to 10 Kilos)  
over a 4 Hour Period.*

Amino acid.	Increase in heat produc- tion per 10 gm. ingested.	Relative effect per gm. ingested (gly- cine = 100).
	<i>per cent</i>	
Glycine.....	29	100
Alanine.....	20	69
Valine.....	None.	
Leucine.....	10	35
Proline	?	?
Hydroxyproline }		
Phenylalanine.....	39	135
Tyrosine.....	21	72
Serine.....	?	?
Cystine.....	8	28
Tryptophane.....	?	?
Glutaminic acid.....	11.5	38
Aspartic acid.....	7	25
Hydroxyglutamic acid.....	?	?
Arginine.....	7-8	25-30
Histidine.....	None.	
Lysine.....	?	?

unknown. It should be emphasized that the limitations of such a tabulation are strict, in so far as the evaluation of the effect of these substances upon the heat production is concerned, and are probably conditioned to a considerable extent by the rate<sub>of</sub> absorption of the individual acids from the alimentary tract. We have, for example, calculated (1927) that per gm. *metabolized* phenylalanine is 2.5 times as powerful in stimulating the cellular energy exchanges as is glycine.

8. *Theoretical Summated Effects of Amino Acids Compared with Effects of Protein.*

With the data now at our disposal we have attempted to see whether the specific dynamic action of the proteins could be accounted for by the summated effects of those amino acids that are known to have a specific dynamic action. The results are to be found in Table XI. In preparing this table, we have made

TABLE XI.

*Theoretical Summated Effect of Amino Acids Compared with Effect of Proteins.*

Amino acid.	Gelatin (7.9 gm. - 6 gm. N).			Casein (44.64 gm. - 6 gm. N).			Gliadin (37.5 gm. - 6 gm. N).			Beef heart (200 gm. - 5.6 gm. N).		
	Amount in gelatin.		Theoretical effect.	Amount in casein.		Theoretical effect.	Amount in gliadin.		Theoretical effect.	Amount in beef.		Theoretical effect.
	per cent	gm.	per cent	per cent	gm.	per cent	per cent	gm.	per cent	per cent	gm.	per cent
Glycine.....	25.5	9.7	29.1	0.45	0.2	0.6				4.0	1.4	4.2
Alanine.....	8.7	3.3	6.6	1.85	0.8	1.6	2.0	0.75	1.5	8.1	2.8	5.7
Cystine.....							0.45			?		
Leucine.....	7.1	2.7	2.7	9.7	3.7	3.7	6.62	2.5	2.5	14.3	4.9	5.0
Phenylalanine...	1.4	0.5	2.0	3.88	1.7	6.8	2.35	0.85	3.4	4.5	1.6	6.3
Tyrosine.....	Trace.			4.5	2.0	4.0	1.5	0.56	1.1	4.4	1.5	3.0
Glutamic acid...	5.8	2.2	2.5	21.77	9.7	11.2	43.66	16.4	18.8	22.3	7.7	8.9
Aspartic acid....	3.5	1.3	0.9	4.1	1.8	1.3	0.58	0.2	0.2	10.6	3.7	2.6
Arginine.....	8.2	3.1	2.3	3.81	1.7	1.3	2.91	1.1	0.8	11.5	4.0	3.0
Total.....			46.1			30.5			28.3			38.7
Found on giving this amount of protein....			38.5			33.3			35.3			39.5

the assumption that the effect of an amino acid is a linear function of the amount ingested. We have already undertaken to point out that under certain circumstances this is not so, at least in so far as the more slowly absorbable acids are concerned. Nevertheless, we believe that with the small quantities involved (see Table XI), where the factor of absorption rate is less in evidence, this error is not a considerable one, and does not invalidate our conclusions.

We have studied in this manner four proteins of widely varying

origin and amino acid content. In the case of gelatin, the protein itself had increased the metabolism 39 per cent; the theoretical increase due to the summated effects of the amino acids was 46 per cent. As to casein, the correspondence is even closer: 33 per cent as opposed to 30 per cent. (These figures may be compared with those in Table VIII.) Gliadin increased the heat production 35 per cent; the theoretical effect of its amino acids was 28 per cent.

In an earlier report (Rapport and Beard, 1927) we stated that the specific dynamic action of beef could be accounted for by the monoaminomonocarboxylic acids. This was an error, due to the unjustified assumption that 25 per cent of the beef heart was dry protein. We have found the average nitrogen content of a large number of samples of freshly cut beef heart to be 2.8 per cent. In other words, the dry protein content of the beef heart we employed was actually 17.5 per cent. Calculating on this basis (Table XI) we find that the theoretical effect of the amino acids in 200 gm. of beef heart would be an increase of 39 per cent in the total metabolism; the actual increase when this amount of the protein was given to our animal was 40 per cent.

It is obvious that these calculations are of necessity only approximations. Moreover, the case is not complete, for certain amino acids have yet to be studied. If one or more of these substances should betray an enormous specific dynamic action on the ingestion of small quantities, the correspondence here shown between the theoretical effect of the amino acids and the actual effect of the proteins would naturally be destroyed. Otherwise, the table may stand, subject to the possible correction of future work, as a fit basis for the generalization that the specific dynamic action of the proteins is fully accounted for by the summated specific dynamic action of their constituent amino acids. In other words, the original hypothesis advanced by Lusk appears to be confirmed.

In an earlier research (Rapport, 1924) it was found that proteins of widely varying amino acid content have about the same influence upon the heat production. The evidence at that time favored the idea that glycine and alanine were the chief contributors, among the amino acids, to the specific dynamic action of the proteins. This conception could not be reconciled with the facts. A search was therefore made for some single unifying cause for the

effect of the proteins on metabolism, as, for example, the synthesis of glycine upon the ingestion of a protein poor in glycine; or the synthesis of polypeptides (capable of stimulating the cellular energy exchanges) during the catabolism of all proteins. But it now appears that the correspondence between the specific dynamic action of the various proteins is, in a sense, accidental. The effect of gelatin is chiefly due to glycine; most of the gliadin effect on the other hand, is due to glutamic acid; while in the case of meat protein, no single amino acid has a predominant influence. The summated effects of the individual amino acids in all of the proteins studied happen to add up to approximately the same figure, but there is at present no basis for attaching a peculiar significance to this.

#### SUMMARY.

1. Both the dicarboxylic acid fraction and the diamino acid fraction of the casein and gelatin hydrolysates increased the total metabolism of a dog.

2. Of the amino acids in these fractions, glutamic acid, aspartic acid, and arginine were found to have a specific dynamic action. Histidine had no influence upon the metabolism.

3. Cystine was also found to have a specific dynamic action.

4. On the basis of the present data, added to those previously established, the actual effects upon metabolism of the proteins are compared with the theoretical effects of the individual amino acids contained in them, and these are found to be in approximate agreement.

5. Certain amino acids have yet to be studied in this connection. Should one or more of these substances prove to have a very large influence on metabolism when ingested in small quantity, the agreement mentioned above might be destroyed. Subject to this possible correction, it is concluded that the specific dynamic action of the proteins is fully accounted for by the summated specific dynamic action of their constituent amino acids.

6. It was incidentally observed that although the weight of the animal studied increased 10 per cent over a 2 month period, the basal metabolism remained constant.

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# THE DESTRUCTION OF VITAMIN E IN A RATION COMPOSED OF NATURAL AND VARIED FOODSTUFFS.\*

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In this paper we wish to report the preparation of a mixed diet, containing several natural food materials, which has been rendered very low in the antisterility vitamin E. This we believe to be of interest not only because of the ease with which the ration may be prepared, thus making a vitamin E-low diet very available, but also for the light which it throws on the properties of vitamin E as found in certain plant tissues.

We were led to undertake the present investigation because of observations made while working on another problem. We were at that time feeding several groups of females various modifications of a stock diet, the object being to determine if young, produced by these females, would have different prenatal stores of available iron.

The stock diet used was a modification of one described elsewhere (1). It had the following composition:

	per cent
Yellow corn.....	71.5
Crude casein.....	5.0
Linseed oil meal.....	15.0
Alfalfa meal.....	2.0
Bone ash.....	1.0

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† E. R. Squibb and Sons Fellow.



	<i>per cent</i>
NaCl.....	0.5
Butter fat.....	5.0
Fresh whole milk <i>ad libitum</i> 6 days of wk.	
Distilled water <i>ad libitum</i> , daily.	

One of the groups of females to which we have referred received the above diet except that to 99 per cent of the dry stock ration was added 1 per cent of ordinary ferric chloride (Powers-Weightman-Rosengarten, iron chloride lumps). In order to secure a thorough mixing and distribution of the iron, the chloride was dissolved in ether (with the aid of a few cc. of water to dissolve the last portions) and poured over the dry stock ration. Sufficient ether was used to cover completely the ration in a porcelain evaporating dish. The ether was then dissipated before a fan at room temperature, the mixture being stirred at intervals until dry.

The females, seven in number, were placed on this diet when about 2 months of age. They continued to grow normally and appeared as vigorous and healthy as other groups carried along at the same time on other modifications of our stock diet. After 2½ months on the iron-supplemented ration a vigorous male was placed in their cage for mating.

It was in reproduction that the iron-fed group showed marked variance from the performance of the other groups. In the course of 2 or 3 weeks, the members of the other groups, the history of which need not be detailed here, mated at the same time as the iron-fed females, showed unmistakable signs of pregnancy. With the iron-fed females these signs were absent. Even after the females of the other groups had borne and suckled generous sized litters, none of the iron-fed females had given birth to young. They continued in this infertile condition until we finally supplemented their diet with small quantities of wheat germ oil and cod liver oil fed in separate dishes. Following these additions the majority of them produced young.

#### EXPERIMENTAL.

In view of the above experience we decided to investigate this problem more fully. In order to do so, however, we substituted a totally dry ration very similar to the dry stock ration and fresh whole milk which the iron-fed females had received before. From numerous consumption records made on our stock females we had

found that about  $\frac{1}{3}$  of the total dry matter consumed was in the form of whole milk. In order to approximate this proportion of intake between dry stock ration and whole milk, we mixed a ration consisting of  $\frac{2}{3}$  stock ration and  $\frac{1}{3}$  whole milk powder.<sup>1</sup>

To one group of females we fed this ration untreated. To a second group of females we fed this ration treated with 1 per cent of ferric chloride. A third group likewise received the iron-treated ration but in addition received individually and in a separate dish 100 mg. of cod liver oil 6 days per week. This group was included to show whether or not the vitamin A content of the iron-treated ration was affected and if so whether or not it was destroyed sufficiently to interfere with normal reproduction. This was particularly necessary in view of the results of Jones (2) who has shown that under certain conditions vitamin A was destroyed in a synthetic ration when ferrous sulfate was included in the salt mixture.

A fourth group of females received the following ration:

Stock ration.....	98.8 per cent	} 5 parts.
Ferric chloride.....	1.2 " "	
Whole milk powder.....		1 part.

In this case the iron was added to the stock ration alone and the whole milk powder was mixed in every day in the above proportions. This was done in order to approximate closely the conditions under which the original group of females was fed, since, it will be recalled, they received fresh whole milk 6 days per week. In their case consumption records showed that they received about  $\frac{1}{3}$  of their total dry matter from the milk. The 1.2 per cent of iron chloride added to the stock ration was sufficient to make the whole mixture contain 1 per cent of iron chloride when the whole milk powder was added and in this way all the iron-fed groups in this series received the same iron intake.

In all the above iron-treated rations, the ferric chloride was added in the manner we have already described; *viz*, by means of ether solution. We also made it a practice not to feed the iron-treated rations before 24 hours, nor after 7 days after the addition of the iron. Thus these rations were made up once a week or oftener.

<sup>1</sup> Klim, Merrell-Soule Company, Syracuse, N. Y.

In order to compare the reproductive performance of animals confined to the above diets with animals fed a purified or synthetic ration such as has been used by Evans and his coworkers, we included two more groups of females. These latter received the following ration:

	<i>per cent</i>
Casein (alcohol-extracted and heated).....	18
Dextrinized starch.....	47
Cod liver oil.....	2
Salts 40 (3).....	4
Lard.....	22
Yeast.....	7

This ration is essentially the same as Ration B.R. 55 of Evans and Burr (4, p. 45), except that we have, instead of feeding a small amount of yeast daily, mixed in 7 per cent of yeast with the other ingredients, replacing 7 per cent of the starch, and have also used our own salt mixture. It may be noted here that Ration B. R. 55 is one of the very low vitamin E diets used by the above workers.

Each of the above six groups contained seven young females from our stock colony. They were placed on the various diets at the time of weaning when they were all between 3 and 4 weeks of age (weight 50 to 60 gm.). All were kept in cages supplied with screen bottoms. The seven individuals of each group were kept in one large cage, except in the case of the group receiving cod liver oil, where they were segregated into smaller individual cages.

All of the animals on the various rations grew well and appeared normal and vigorous.

After approximately 9 weeks on these rations (85 to 90 days of age) when the females were well grown (close to 200 gm. or over) vaginal smears were made daily according to the technique of Long and Evans (5). Shortly thereafter a vigorous male was placed in each cage. By means of daily vaginal smears the onset of estrus was noted and any positive mating following this was invariably established by the finding of the vaginal plug or residual sperm. After a positive mating by any female the daily vaginal examination was discontinued on that animal for 4 days and taken up again on the 5th day and continued until the placental sign (red blood cells) was shown, or, if the female proved not

pregnant, until she mated again. Beginning several days before or on the 14th day after mating daily weighings were made of each animal and continued until she had given birth to young or until well past the 21st day. All pregnant individuals were segregated on the 19th or 20th day of gestation in separated cages

TABLE I.  
*Record of First Gestation on Various Diets.*

Group No.	Diet.	No. of animals showing positive mating and placental sign.	No. of litters born.	Total No. of young born.	Initial fertility.
	<i>per cent</i>				<i>per cent</i>
I	Stock ration..... 66½ Whole milk powder.... 33½	7	7	62	100
II	Stock ration + whole milk powder..... 99 Ferric chloride..... 1	7	0	0	0
III	Same as for Group II + 100 mg. each cod liver oil 6 days per wk.	7	0	0	0
IV	Stock ration..... 98.8 Ferric chloride..... 1.2 Whole milk powder.... 1 part.	5	0	0	0
V	Casein (extracted).... 18 Starch..... 47 Cod liver oil..... 2	5	5	41	100
VI	Salts 40..... 4 Lard..... 22 Yeast..... 7	6	6	48	100

supplied with shavings litter. Females bearing young were not allowed to suckle them.

In this way we collected accurate data on the reproductive functions of the six groups of females receiving the various rations outlined. In Table I we present these data. It will there be seen that the females of Group I receiving the untreated mixture of stock ration and whole milk powder produced in every case a

generous sized litter. The females of Groups II, III, and IV, those receiving iron-treated rations, uniformly showed resorptions, giving birth to no young whatever. In Group IV, two of the females did not ovulate, showing throughout a period of 3 or 4 weeks a vaginal smear characterized by very large numbers of leucocytes, suggesting an infection of the genital tract. They were therefore discarded. The females of Groups V and VI, those receiving a synthetic ration of more or less purified ingredients, a ration presumably low in vitamin E, all nevertheless bore young. Two females in Group V and one in Group VI did not become pregnant during the course of time required to gather these data and hence are not included in Table I.

In Chart I we present the weight charts of the females of Groups I and II to show in the two cases the typical drop when young are born as opposed to the gradual drop during resorption of young. These records are exactly typical of our data.

We were very much surprised at the uniform behavior of the animals on the various dietaries. Evans and Burr, in chapter IV of their monograph (4) and elsewhere (6) have emphasized the fact that first gestation fertility ("initial" fertility) was encountered in some degree on nearly all rations investigated by them. Only when they make use of very highly purified and restricted diets, as for example, their Diet 519 (casein purified, 25; sucrose recrystallized, 75; salts, 4), do they succeed in getting complete sterility. They have explained this phenomenon of initial fertility on the assumption that young animals are born with considerable stores of vitamin E which are slowly dissipated by the various metabolic processes, the stores being sufficient, however, to carry a certain number of females successfully through the first gestation, especially when there are traces of vitamin E still present in the ration.

We expected, therefore, some fertility in all of the groups during the first gestation. The fact, however, that all of the females receiving the iron-treated rations were sterile while those receiving the synthetic ration showed such high fertility suggests that the iron-treated rations are very low in vitamin E content.

Although the performance of the females on the iron-treated rations was in every way indicative of lack of vitamin E we decided to cover this point further and see if they would now re-

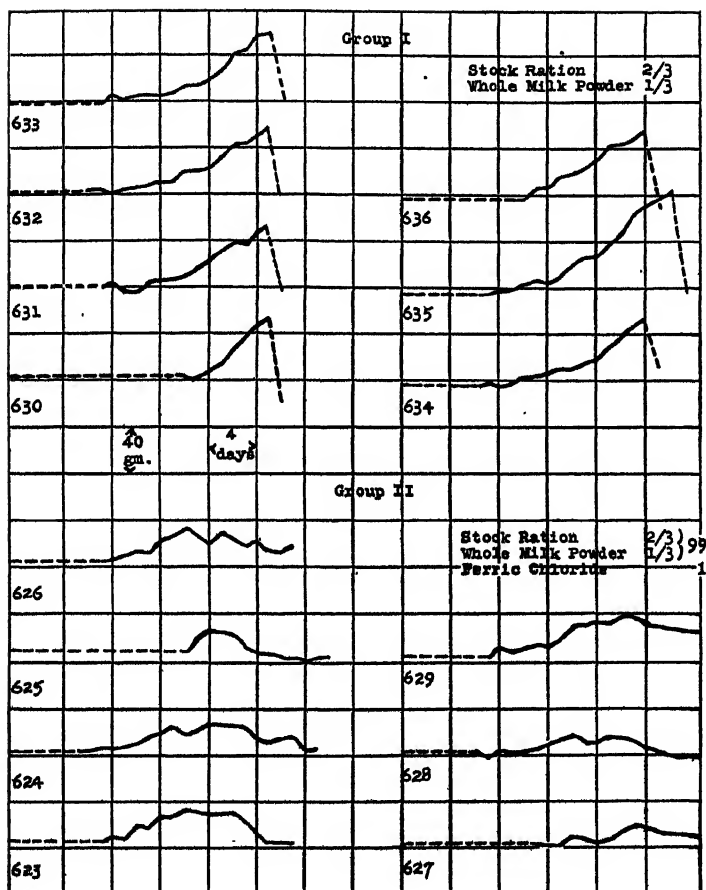


CHART I. There are presented here the curves of body weight of the females of Groups I and II for the first gestation period. In Group I, those fed the untreated ration, there can be noted the steady increase in weight with the precipitate drop on the day of littering; in contrast, the females of Group II, receiving the iron-treated ration, show the rounding curve of resorption. The curves in all cases start with the day of positive mating; the solid portion representing the period of daily weighings.

spond to vitamin E therapy. This was particularly desirable since it might be argued that the relatively high iron intake had wrought a destructive effect on the reproductive organs. To this end, therefore, we took the nineteen females which had received the iron-treated rations and fed them the following rations during the second gestation.

TABLE II.

*Second Gestation Performance of Females, Which on Various Iron-Treated Rations Had Had First Gestation Resorption.*

Diet.	No. of animals showing positive mating and placental signs.	No. of litters born.	Total No. of young born.
<i>per cent</i>			
Stock ration + whole milk powder..... 98	3	3	21
Wheat germ oil..... 2			
Stock ration + whole milk powder.....	4	4	29
Stock ration + whole milk powder..... 99	3	3	33
Ferric chloride..... 1			
Wheat germ oil, 200 mg. each 6 days per wk.			
Casein (extracted)..... 18	4	0	0
Starch..... 47			
Cod liver oil..... 2			
Salts 40..... 4			
Lard..... 22			
Yeast..... 7			
Stock ration + whole milk powder..... 99	3	0	0
Ferric chloride..... 1			

Three were changed to the untreated mixture of stock ration and whole milk powder fortified with 2 per cent of wheat germ oil. Four were placed on the untreated mixture alone. Four were continued on the iron-treated mixture of stock ration and whole milk powder but each received in addition 200 mg. of wheat germ oil 6 days per week in a separate dish. Four were

changed to the synthetic ration in order to see if this ration contained sufficient vitamin E to bring about fertility in females which had had resorptions. The remaining four females were continued on the iron-treated mixture of stock ration and whole milk powder.

Vaginal smears were made daily and the females mated as before. Data on the second gestation performance were thus secured. These data are summarized in Table II. One of the four females receiving the iron-treated ration plus wheat germ oil ovulated and mated with regularity but failed to become pregnant. Another female among those receiving the iron-treated ration alone exhibited this same phenomenon. These two animals are, therefore, not included in Table II.

Of the females comprising Groups V and VI, which had received the synthetic ration, ten were continued through the second gestation on that ration unchanged. Their performance in this case was that of uniform lack of fertility, all showing typical resorptions.

A study of Table II will show that the untreated mixture of stock ration and whole milk powder, either alone or fortified with 2 per cent of wheat germ oil, enabled the sterile females to bear generous sized litters. This was also true of those females which received the iron-treated mixture supplemented with wheat germ oil fed separately. These results indicate very convincingly that the first gestation sterility was due to lack of vitamin E. In contrast to the above performance it should be noted that those sterile females which received the synthetic ration, as well as those which were continued on the iron-treated ration, remained sterile and repeated their first gestation performance.

#### *Vitamin A Content of Iron-Treated Mixture of Stock Ration and Whole Milk Powder.*

Because of the well known fact that vitamin A is susceptible to oxidative destruction and that iron salts are excellent catalysts we were naturally interested in finding out if the iron-treated mixture was noticeably deficient in vitamin A. Concurrently therefore, with the first experiments on the vitamin E content of this ration we also investigated its ability to cure rats suffering from ophthalmia. The animals used for this experiment had been kept for a period of 4 or 5 weeks on a basal synthetic diet (7)



which had been irradiated for 30 minutes under a quartz mercury vapor lamp. They were, therefore, suffering from low vitamin A uncomplicated with lack of antirachitic factor. They were taken at a time when the ophthalmia was at the erythemic stage and fed (a) the iron-treated ration alone, (b) the iron-treated ration mixed with an equal quantity of the basal synthetic ration, and (c) the iron-treated ration mixed with 3 times the quantity of basal synthetic ration.

Three animals received the iron-treated ration alone. Five animals in each case were used to test the two mixtures of iron-treated and basal synthetic rations. In the course of 2 weeks marked improvement in the eye symptoms of all these animals was noted and growth had been resumed. At the end of 3 weeks in every case the ophthalmia had been completely cured.

These results indicated, therefore, that the iron-treated ration contained sufficient vitamin A to cure ophthalmic rats even when making up only 25 per cent of the ration. In addition to this it may be stated that our experience with the females receiving this ration from the time of weaning until maturity had given us no indication of lack of vitamin A. The growth and well-being of these animals has been in every way comparable to those receiving the untreated mixture. Quite recently Evans and Burr (8) have shown that lack of vitamin A brings about a characteristic disturbance of the reproductive apparatus of the female rat, this disturbance being characterized by a continuous vaginal smear of cornified cells. Nothing of this nature was observed by us in the females confined to the iron-treated ration.

From all these considerations it seems justifiable to state that the vitamin A content of the iron-treated mixture of stock ration and whole milk powder is not greatly affected. It may also be stated that the palatability of the ration is apparently not appreciably diminished.

#### DISCUSSION.

The experiments which we have outlined above indicate that the vitamin E contained in the mixture of stock ration and whole milk powder is destroyed or inactivated by the iron treatment. Hopkins (9) showed quite early that vitamin A contained in butter fat was destroyed by aeration and more recently Jones (2)

has shown that the vitamin A of butter fat may be destroyed under certain conditions in a synthetic ration. The presence of ferrous sulfate hastened the destruction. Quite recently also data have appeared which suggest that vitamin E may also be quite labile under conditions favoring oxidation. Thus Evans and Burr (10) have found that lard, Crisco, and oleic acid, when mixed with minimal doses of wheat germ, render it impotent in the cure of sterility. Mattill (11) as a result of his work on the oxygen absorption of certain fats and oils states that the oxidative changes that accompany the development of rancidity in unsaturated animal fats tend to destroy vitamins A and E. More recently Evans and Burr (6) as an explanation of their previous results have postulated the presence of antivitamin substances in some fats, which increase with rancidity.

Undoubtedly in the iron-treated ration which we have described there are oxidative changes going on. Although the ration does not become markedly rancid during the course of the week in which each batch is fed, it has, nevertheless, a slight acrid odor which the untreated ration does not have. The rancidity is not sufficient to affect markedly the palatability since the consumption has always been good.

A point of interest lies in the fact that the vitamin E contained in such ingredients of the ration as yellow corn, linseed oil meal, whole milk powder, *etc.*, should be so completely destroyed while the vitamin A contained in the same materials largely escapes destruction. Apparently, therefore, the vitamin A in such tissues is either more resistant or better protected than is the vitamin E. This assumption seems particularly tenable in view of the lability of vitamin A in fats and oils (Hopkins, Jones) to oxidation as contrasted to the stability of vitamin E in wheat germ oil. This stability has been shown by Evans and Burr (6) who aerated wheat germ oil at 100° for 10 hours without noting any deterioration in vitamin E potency.

Whatever may be the explanation of the results secured, which future work must show, it seems clear that our experiments point to a very complete destruction of the vitamin E potency of a diet composed of natural and varied foodstuffs by treatment with an ether solution of ferric chloride. Whether or not the storage reserves of an animal are affected remains to be seen.

## SUMMARY.

1. It has been shown that the vitamin E potency of a ration composed of natural and varied food materials may be completely destroyed by treatment with ferric chloride.

2. The vitamin A content of this iron-treated ration is not appreciably affected nor is the palatability greatly reduced.

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## DISTRIBUTION OF UNSATURATED FATTY ACIDS IN TISSUES.

### III. VITAL ORGANS OF BEEF.

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The unsaturated fatty acids, especially those of the higher degrees of unsaturation, appear to be of great importance in the part which the lipids play in cell life. It was believed therefore that a study of the distribution and character of these substances in tissues, including the types of compounds in which they were contained and the degree of their unsaturation, was a necessary preliminary step in the study of their functions. Data have been presented on the heart (1) and other muscles (2) of beef, and in the following are contained similar data on the more important vital organs of the same animal. As noted previously, attention has been confined to the tissues of one animal because it was believed that only in that way could the relative importance of the different lipids be established. An animal was chosen of such size and availability that large amounts of tissues could be easily obtained, allowing the use of standard gross methods of separation and analysis and avoiding in this way assumptions as to the nature of the compounds studied. No account was taken of age or similar variables since it was desired at first to find out the extent of variations in normal animals. Conditions were standard and comparable in that the animals had been prepared for slaughter and sale in the good markets of the city and were therefore presumably normal and healthy and had gone through a period of fattening and hence relative rest and good nutrition.

*Procedure.*—The tissues were received while still warm from the animal and the examination was begun at once. The procedure for the extraction and examination was the same as in the preced-

ing publications on muscle (1) and need be given only briefly. It consisted in complete extraction of the hashed tissue by continuous extraction with hot 95 per cent alcohol, removal of the solvent by distillation at low pressure and temperature, separation of the phospholipids (lecithin and cephalin) by double precipitation with acetone from ether solution, separation of lecithin from cephalin by precipitation of the latter from ether with absolute alcohol. In most cases the acetone-soluble fraction containing the ordinary fat was first freed from such amounts of phospholipid as could be precipitated by  $MgCl_2$  (which was examined separately) and then analyzed. No distinction was made in the tables between the acetone-soluble fraction so treated with  $MgCl_2$  and that which was not, since the amount of material so separated was generally small and appeared to make little difference in the composition of the acetone-soluble fraction. The analysis comprised (1) weights of each of the fractions and of the fatty acid constituents after saponification, (2) iodine absorption values (iodine numbers) of the intact lecithin and cephalin, of the total fatty acids, and liquid fatty acids (separated by the Twitchell procedure) of all the fractions, (3) formation and separation of the bromine addition products of the unsaturated acids with especial attention to the hexa- and octobromo compounds.

Completeness of extraction of the tissues with the hot alcohol was tested from time to time by taking an aliquot portion of the completely extracted material, digesting it for 8 hours or longer on the water bath with 20 per cent alkali, acidifying, extracting with ether, purifying the ether extract by evaporating to dryness and dissolving with petroleum ether, then evaporating the petroleum ether, drying, and weighing the residue. The weight of the residual extract was never more than 5 per cent of the total extract and was generally less than 2 per cent.

The whole series of processes was ordinarily carried through within a week and precautions were taken to minimize oxidation by keeping the extracts in the cold and dark when not in actual process of examination and by the use of carbon dioxide to displace the air during heating, except in the processes of extraction in which the vapors of the solvent were believed to serve the same purpose.

The results of the examinations are given in Table I.

## DISCUSSION.

*Contents of Lipids in Tissues.*—As may be seen from Table I, the organs showed quite wide variations both in the different kinds of organ and in different organs of the same kind. The constituent which was most constant for each kind of organ was the total phospholipid with a maximum variation of 30 per cent from the average value. When the organs were arranged in the order of their content of this constituent, the brain showed the highest content, the liver next, then pancreas, kidney, and lung in order. This arrangement is the same as found for the phospholipid and cholesterol content of the organs of different small animals by Mayer and Schaeffer (3) and Terroine and Belin (4). In the case of the various muscles of beef it was found that the phospholipid content ran parallel with the probable activity of the muscle—those muscles which were more continuously and strongly active having a higher phospholipid (and unsaponifiable) content than those which were less active. The same conception may be applied with limitations to the organs. The brain, which has the greatest variety and extent of function—that of controlling all the activities of the organism—has the highest content of phospholipid and cholesterol. The liver, which is next in importance in the successful functioning of the organism, comes next to the brain in its content of phospholipids, then the pancreas with two important secretory functions, then the kidney and lung, each with one important function. The relation between function and the unsaponifiable fraction observed in muscles, apparently does not hold for the organs, although the brain, which had the highest content of phospholipid had the highest content of unsaponifiable fraction. Function or activity in this connection must be taken to mean all those activities which go to make up life processes in the cell and not metabolic or energy-producing activity exclusively. (In fact the muscles, which are the main seat of metabolic activity, fall, with the exception of the heart muscle, below the organs in their content of phospholipid.) In this sense there appears to be a definite relation between phospholipid content and function in the organs.

Mayer and Schaeffer (3) and Terroine and Belin (4) found that the content of phospholipid and the content of cholesterol ran parallel in tissues. In the results reported in Table I the un-

TABLE I.—*Lipids*

Cephalin.	Liver.				Kidney.			
	High.	Average.	Low.	Type.	High.	Average.	Low.	Type.
Weight, gm. per kilo.....	27.00	15.00	8.90	13.45	11.50	7.43	6.30	6.8
Iodine No.....	96	80	67	83	100	88	74	95
Total fatty acids, per cent.....	67	48	58	63	73	65	58	59
Iodine No.....	132	119	100	112	136	109	100	138
Solid fatty acids, per cent of total fatty acids.....	26	18	11	16	27	18	12	14
Liquid " " " ".....	52	40	26	50	61	51	34	60
Iodine No.....	217	179	134		192	179	165	182
Unbond acid, per cent of liquid fatty acids.....	34	19	12	20	14	11	9	11
Lecithin.								
Weight, gm. per kilo.....	20.00	15.64	6.8	20.95	13.20	8.78	5.63	8.7
Iodine No.....	98	83	71	77	93	85	73	91
Total fatty acids, per cent.....	74	65	55	70	73	72	66	69
Iodine No.....	127	108	83	93	126	110	97	113
Solid fatty acids, per cent of total fatty acids.....	30	20	6	13	38	21	10	21
Liquid " " " ".....	64	46	31	39	51	43	35	48
Iodine No.....	193	160	124	132	183	156	132	160
Unbond acid, per cent of liquid fatty acids.....	30	21	13	21	19	12	9	10
Acetone-soluble (fat).								
Weight, gm. per kilo.....	12.60	9.00	4.30	19.25	16.35	9.00	6.53	7.1
Total fatty acids, per cent.....	78	70	52	78	89	85	74	87
Iodine No.....	117	87	53	53	114	82	63	65
Solid fatty acids, per cent of total fatty acids.....	51	33	15	51	52	34	25	27
Liquid " " " ".....	53	44	30	34	52	46	33	33
Iodine No.....	158	132	103	98	155	136	121	123
Total phospholipid, gm. per kilo...	38.90	30.80	21.05	34.40	21.15	16.18	10.66	15.1
Unsaponifiable fraction " " " ...	3.61	2.18	1.04	2.78	2.37	1.88	1.06	1.1

# W. R. Bloor

## of Beef Organs.

Pancreas.				Lung.			
High.	Average.	Low.	Type.	High.	Average.	Low.	Type.
11.35	8.15	5.82	10.35	6.58	5.68	3.30	6.33
79	69	57	70	85	78	64	76
71	61	51	67	74	63	51	68
11	98	82	110	116	104	84	93
30	16	6	10	39	21	9	30
77	51	29	56	59	48	33	49
62	146	135	162	191	152	132	144
9.6	7	5	7	15	9.7	3	12
13.15	10.49	8.5	13.15	9.80	6.80	4.90	5.23
79	68	62	76	82	73	57	74
81	69	58	76	77	69	58	70
99	91	67	94	103	93	84	87
20	13	7	10	35	18	10	35
57	46	34	44	62	47	42	42
57	138	116	138	158	136	108	129
6	5	4	6	19	11.4	6	11
37.04	39.04	16.90	23.60	11.50	7.56	3.58	8.53
99	86	76	81	92	75	58	58
72	62	55	63	91	76	58	62
47	31	21	35	38	27	14	37
34	44	28		62	50	33	48
27	109	101	112	126	114	101	103
23.50	19.30	14.00	23.50	15.58	11.90	9.00	11.56
3.50	1.29	0.70	1.84	3.21	2.47	1.78	3.09





saponifiable fraction, which includes cholesterol, does not run parallel with phospholipid but since unsaponifiable contains much substance which is not cholesterol this does not invalidate the findings of these workers. As noted the unsaponifiable fraction of the brain, which is nearly pure cholesterol, is the highest of all the organs. As was found for the muscles, the weights of lecithin in each tissue were not markedly different from those of cephalin although the weight of cephalin was always higher. As shown by the analysis and as has been pointed out by earlier workers, the cephalin fraction probably contained considerable impurities, so that the true weight would be still nearer that of lecithin. The possibility of an equimolecular combination of lecithin and cephalin in tissues suggested by the work on muscle (1) and by the synthetic work of Grün and Limpacher (5) is thus indicated for the organs also.

As may be seen from Table I, quite wide differences in values of the fractions were found in different samples in all organs. The greatest variations were found in the acetone-soluble (fat) fraction where a spread of values between highest and lowest of 200 to 500 per cent of the lowest value was found. The unsaponifiable fraction came next in variability, the differences being slightly less than in the acetone-soluble fraction. Lecithin and cephalin were found to be less variable and the sum of the two as total phospholipid was least variable of all, the spread of values not exceeding 100 per cent of the lowest value and 30 per cent above or below the average. The liver showed the widest spread of values for lecithin and cephalin individually, a fact which may perhaps be referred to a special function of the liver.

With regard to the causes of these variations, it may be noted that fat is a stored substance and its variations probably have little immediate significance in cell processes. The unsaponifiable fraction is a mixture containing, as shown by Lemeland (6) and Anderson (7), cholesterol, other sterols, and related substances and various unknown substances among which are probably residues of oxidative changes in the unsaturated fatty acids. The relative importance of the sterols is unknown although much attention has been paid to cholesterol. It is obvious that this group must be separated into its constituents and these examined separately before their significance is understood. As regards

lecithin and cephalin, it was shown by MacLean (8) that lecithin may be changed by certain treatment into a substance having the solubilities of cephalin so that the relatively large differences sometimes found in these two substances may perhaps be due to some change of this kind.

*Percentage of Fatty Acids in the Compounds.*—A palmitic-oleic acid lecithin should yield 69 per cent of its weight of fatty acid; a similar cephalin, 76 per cent. Since palmitic and oleic are the acids found in greatest quantity in the phospholipids, these values may be taken as average values. A similar fat, dipalmito-olein, would yield 95 per cent of its weight as fatty acid. If these theoretical values are compared with those found in the analyses reported in Table I, it will be noted that the fatty acid values for lecithin in the organs, except the brain, are very near the theoretical values. In the brain lecithin, the fatty acid percentage was about 9 per cent higher and in the muscles about the same per cent lower, but on the whole the values indicate that the lecithin as separated was fairly pure. The fatty acid percentage in the cephalin fraction was, however, in all cases much below the theoretical value,—in brain 10, liver 24, pancreas 20, kidney 20, lung 17, heart 13 per cent, and in the other muscles averaged nearly 30 per cent too low, indicating that the cephalin fraction as separated contained a great deal of substance which was not cephalin, a finding which is in agreement with the statement of MacLean (8) that cephalin as usually prepared is not a single individual but a mixture of cephalin with its degradation products. Even on a relatively pure sample of brain cephalin, Levene and West (9) found only 64 per cent fatty acid instead of the expected 75.9 per cent.

The percentage recovery of fatty acids from the "fat" fractions in the case of the organs was also much below the theoretical, being nearer the theoretical only in the case of the pancreas where the amount of fat was relatively high. These results indicate that the fat (acetone-soluble) fraction also contained much material that was not ordinary fat and which, in part at least, may be acetone-soluble phospholipid. A small amount of material may be precipitated from the acetone by the addition of  $MgCl_2$  and its analysis indicated that it is closely related to the phospholipids, but the remainder after removal of the  $MgCl_2$  was not

markedly different in its analysis from the whole fraction so that there was still much non-fat present in this fraction. These differences from expected values for the lipids constitute one of the problems which this preliminary survey has exposed.

The liquid fatty acids were found, as in the muscles, to average about half of the total fatty acids and there was again the marked discrepancy (ordinarily more than 25 per cent) between the sum of solid and liquid fatty acid percentages and the total fatty acids, indicating a large and probably important fraction of the fatty acids at present unaccounted for.

*Iodine Absorption Values.*—The iodine absorption value (iodine numbers in Table I), being a measure of the degree of unsaturation of the fatty acids, has generally been considered a criterion of the oxidizability and perhaps of other functional powers of the lipids. Its determination should be of especial value in the study of the function of the lipids. While it is now realized that the present methods of separation and examination probably do not sufficiently protect the unsaturated acids from change (see "Oxidation" below), so that the values found are to be regarded as minimum values, the series of processes was the same for all samples and the results should be comparable. The iodine numbers of the lecithin and cephalin especially were taken at once after separation and thus after only a short period following their removal from the animal. They should represent fairly closely the values as they would be in the intact material of the tissues. Again, although the iodine values for the separated fatty acids are apparently lower than they should be, they run parallel with those of the intact lipids and so may be compared with each other.

When the tissues are arranged in the order of the iodine values of the phospholipids, brain is highest, kidney and liver next and close together, then lung, with pancreas at the bottom of the list. Nevertheless, the difference between the iodine numbers of the phospholipids of the organs (and the muscles) (2) was in most cases not great, (the pancreas was the only wide variant) and points to a considerable basic content of the same phospholipid in all tissues. In the fat fraction (brain had no appreciable fat fraction) liver fat had the highest iodine number, then kidney,

then lung, and finally pancreas, which was the only organ with anything more than a small amount of fat.

In the muscles (2) the iodine numbers of the phospholipids were irregular, but in general they fall in the same class as the liver and kidney. As regards the fat fraction of the muscles, the iodine values indicated that the fat present was mainly the same as the stored fat of this animal.

The iodine numbers of the fatty acids of the phospholipids were as usual always much higher than those of the fatty acids from the fat fractions. Of the phospholipids themselves, the iodine numbers of the intact compounds were very similar, although slightly higher for the cephalin. But, as would be expected from the fact that the fatty acid percentage in the cephalin is much below theoretical, the separated fatty acids of the cephalin had a higher iodine number than those of the lecithin, a difference which is also shown in the liquid fatty acids. The difference in iodine numbers between lecithin and cephalin liquid acids averaged, however, only 12 per cent in both organs and muscles. This fact is of importance since it shows that, as regards the nature of their fatty acids, the two phospholipids are not very different and no essential mistake would be made in considering them together, as is frequently done.

As regards the iodine numbers of the fatty acids of the fat fractions, compared with the iodine number of the stored fat of this animal (about 65) they are distinctly higher only in the liver, kidney, lung, and heart muscle and notably higher only in the liver and kidney, in both of which the percentage yield of fatty acids is very low—lowest of all the tissues and within the range of percentage content of the phospholipids. The inference is that a large portion of the fat fraction in these two organs is phospholipid.

*Oxidation.*—The iodine values of the fatty acids from lecithin and cephalin were always lower than the calculated theoretical values, if the percentage of fatty acids in the phospholipids is taken into account. This difference probably indicates that oxidation had taken place during the separation, although there is a possibility that the phosphoric acid-choline combination favors the taking up of more iodine by catalyzing absorption or perhaps replacement. As further evidence of oxidation a similar difference between theoretical and found iodine values was found

in benzene and this must include some of the 4-bond addition products which are not absolutely insoluble in this solvent. This finding agrees with that of Theis on beef liver (10) and with our own work on muscle (2). Whether this means that the 3-bond acid was not present in the original tissues or that it was present and was destroyed or changed in the separation, remains to be seen. The 25 per cent of fatty acids found missing in the separation into solid and liquid fractions may have something to do with it. The 4-bond acid, presumably arachidonic, was present in fairly large amounts. Thus for cephalin the values were: liver 12 to 34, average 19 per cent of the liquid acids; lung 3 to

15, average 9.7 per cent of the liquid acids; pancreas 5 to 9.6, average 7 per cent; for lecithin: liver 13 to 30, average 21 per cent; pancreas 4 to 6, average 5 per cent; lung 6 to 19, average 11 per cent, of the liquid acids.

*Lipids of Liver.*—Since the liver enters largely into all considerations of fat metabolism and since analytical data on beef liver lipids by the same procedure as the above have recently been published by Theis (10), a special discussion of the two sets of data seemed called for. Theis' findings on normal beef livers may be compared with our own, as to significant details, by Table II.

The greater total weight of lipid in Theis' samples was obviously due to a higher fat content, since our phospholipid percentage was higher than his. This fact is further brought out by the weights and percentages of the acetone-soluble (fat) fraction. These differences between our results and Theis' in content of fat in the liver are probably to be regarded as normal, since the fat content of the liver is recognized as variable. Theis' iodine numbers are throughout higher than ours, a fact which is due probably to better technique in preventing oxidative changes. The same difference is reflected in his much higher values for 4-bond acids.

The percentage recovery of fatty acids both from phospholipid and (fat) fractions is practically the same as ours and emphasizes the fact mentioned above that these fractions must contain a good deal of material which is not ordinary phospholipid or fat, respectively. This remark applies with especial force to the "fat" fraction, in which the fatty acid recovery is about 25 per cent too low, and constitutes a problem which must be solved before much can be said about fat as such in the liver or other organs.

#### SUMMARY.

The lipid content of the organs varied greatly both for the same organ and for different organs. The constituent which varied least for each organ was the total phospholipid, which was relatively constant, not varying more than 30 per cent above or below the average value. It may therefore be regarded as a tissue constant characteristic of the particular organ. The arrangement of the organs in the order of their phospholipid content gave a series which represented the order of their functional activity.

The similarity in the weights of lecithin and cephalin in the various samples of all tissues points either to an equimolecular equilibrium between the two, or to an equimolecular combination between them.

The average iodine numbers of lecithin and cephalin of each organ were so close to each other that a close similarity between these two compounds was strongly indicated.

Although certain organs rate higher in iodine number of phospholipid than others, the difference was not great, and this similarity points to a considerable basic content of the same phospholipids in all organs and tissues.

The percentage content of fatty acids in the fractions as separated indicated that the lecithin fraction was quite pure, but that the cephalin and fat fractions contained considerable admixtures of other substances.

The mixture of fatty acids obtained by saponification of the various fractions consisted, as in the muscles, of about one-half liquid or unsaturated acids and about one-quarter solid acids, the remaining quarter being unaccounted for.

The unsaturated acids contained relatively large amounts of a 4-bond acid, probably arachidonic, but no appreciable amount of 3-bond acid. The amount of 4-bond acid was greatest in the brain, next in the liver and kidney, then lung, then pancreas.

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# THE HIGHLY UNSATURATED FATTY ACID OF LIVER LIPIDS.

## THE PREPARATION OF ARACHIDONIC ACID.

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The presence of arachidonic acid,  $C_{20}H_{32}O_2$ , in the lipids of liver was demonstrated by Hartley in 1909 (1), his evidence being the isolation of octobromoarachidic and octohydroxyarachidic acids. Since that time a number of investigators have identified this acid as a constituent of various tissues. Levene and Simms (2) and Levene and Rolf (3) inferred its presence in brain cephalin and lecithin and in liver and egg lecithin. They reduced the octobromides from brain cephalin in boiling alcohol with zinc and HCl. The product was an oil, which upon bromination yielded an insoluble bromide containing 67.42 per cent bromine (theory 67.78). Their product from liver lecithin had an iodine number of 305. Wesson (4) verified the presence of the acid in brain lipids. His debromination was carried out by treatment of the bromides with copper-covered zinc dust in alcohol, the product being described as a colorless or slightly yellow oil of tenacious fishy odor which upon hydrogenation yielded arachidic acid. Cartland and Hart (5) obtained octobromoarachidic acid from the unsaturated fatty acids of corpus luteum. Upon treatment with zinc and alcohol containing hydrochloric acid a specimen of arachidonic acid was obtained which gave an iodine number of 309 (theory 334). Eckstein (6) and Wagner (7) proved the presence of arachidonic acid in small quantities in human depot fat. Eckstein's bromides analyzed satisfactorily for octobromoarachidic acid, but the arachidonic acid obtained by reduction gave an iodine number of only 288. The presence of arachidonic acid in the lipids of beef heart was inferred by Bloor (8). The significance of the acid in metabolism and factors influencing its content in the tissues have been discussed by Wesson (9).

The object of the present work was twofold; first to verify the presence of arachidonic acid in the lipids of liver, and second to isolate a specimen of the pure acid. As regards the first, the methyl esters of the fatty acids of liver lipids were prepared and fractionated into four fractions, and each fraction analyzed.

The bromides of each fraction were found to contain practically the same amount of bromine, the results in each case being very close to the theoretical for methyl octobromarachidate. These results indicate, therefore, that arachidonic acid is the sole highly unsaturated fatty acid present in appreciable quantities. As regards the second, the combined bromides of the several fractions were reduced with zinc in neutral alcohol, and practically pure methyl arachidonate was prepared. From this ester the acid was obtained by saponification. While some oxidation took place during the saponification, the final product of iodine number 316 represents the purest specimen so far obtained. The properties of the ester and acid are described.

#### EXPERIMENTAL.

*Liver Lipids.*—The writer is indebted to Dr. David Klein of the Wilson Laboratories for a supply of the lipids of pig livers. These were prepared from desiccated liver (dried in a vacuum at low temperature) by extraction with benzene and subsequent removal of the solvent by distillation under reduced pressure. The product was a chocolate-brown waxy material at ordinary temperature.

*Saponification.*—600 gm. of lipids were melted in a 5 liter flask and warmed to about 60°. A hot solution of 400 gm. of KOH in 400 cc. of water was added. While being vigorously shaken, 350 cc. of alcohol were gradually poured into the flask. Saponification took place at once and was complete in 5 minutes. The soaps and other material present were dissolved in 2 liters of hot water. The fatty acids were liberated by cautious addition of an excess of HCl. The contents of the flask were boiled until the fatty acids formed a clear layer. Upon separating, the yield of crude acids was 553 gm.

*Preparation of Methyl Esters.*—The crude acids were dissolved in 1200 cc. of absolute methyl alcohol containing about 1 per cent of dry HCl gas and refluxed for 22 hours. About a third of the alcohol was then distilled, the remainder cooled, and poured into 2 liters of cold water. Addition of a liter of ether facilitated separation. The ether solution was transferred to another flask and the ether removed by distillation. The esters were distilled quickly at 8 to 10 mm. pressure. Yield 500 gm., boiling at 180–230°, light amber in color.

*Distillation and Analysis of Methyl Esters.*—475 gm. of the esters were distilled from a 1 liter Claissen flask at 5 to 6 mm. In order to make a more complete separation of the low and high boiling constituents the distillation was carried out slowly for the first two fractions and more rapidly for the last two.

The fractions were analyzed by the usual methods (Table I). The mean molecular weight of the acids was calculated from the neutralization equivalent after saponification with alcoholic potash, by subtracting 14 ( $\text{CH}_2$ ). The iodine number was determined by the Hanus method. The polybromide number was found by brominating a weighed sample in ether, allowing it to stand at least 4 hours, and subsequent washing at least four times, with ether, followed each time by centrifugation. The bromine

TABLE I.  
*Analysis of Fractions.*

Boiling point, 5 to 6 mm.	Weight.	Mean molecular weight of acids.	Iodine No.	Free acid as oleic.	Polybromide No.	Bromine in bromide.
°C.	gm.			per cent		per cent
165-175	97	260.4	45.31	1.0	1.9	66.78
175-190	204	266.0	47.27	1.2	3.2	65.97
190-200	103	271.8	77.80	1.7	6.2	66.80
200-235	63	292.8	110.3	3.5	20.0	66.36
Total.	467					
Original mixed esters.		274.9	71.85	1.4	6.0	

determination was made by the Parr peroxide bomb method. Further details and discussion of these methods have been given by the writer previously (10).

*Preparation of Methyl Arachidonate.*—29 gm. of methyl octobromoarachidate, obtained by brominating the fractions above and those from a duplicate experiment, were suspended in 250 cc. of methyl alcohol and refluxed for 24 hours with 50 gm. of zinc powder. The alcohol solution was poured off while still hot and the residue in the flask reextracted with 200 cc. of boiling methyl alcohol. The combined alcohol extracts were centrifuged to remove suspended solids, and most of the alcohol was distilled. The residue was poured into water containing a little HCl to decompose any zinc soap present. The oily layer was shaken out

with ether and separated, these operations being carried out as rapidly as possible and, where feasible, under  $\text{CO}_2$  gas. The ether was removed under reduced pressure and the ester distilled. The yield was 7.5 gm. of methyl arachidonate, boiling at  $200\text{--}210^\circ$  at 7 mm. pressure. Yield 77 per cent of theory. The product was a very light amber-colored oil with a fishy odor. Upon exposure to air the ester slowly hardened to a transparent film. Iodine number (Hanus 3 hour) was 314.1 (theory 319.2). When allowed to stand for 18 hours with the Hanus reagent the iodine number was higher than the theoretical, 329. The molecular weight of the ester as determined by saponification was 312 (theory 318). This result was subject to considerable error, due to the small quantity used in the determination, 0.26 gm. The polybromide number of the ester was found to be 77.6 (theory 201). This wide discrepancy is however in excellent agreement with results obtained on the methyl esters of the highly unsaturated acids of menhaden oil (10) and also on other unsaturated acids such as linolenic which gives a hexabromide number of only 50 as compared to a theory of 172.  $n_D^{25} = 1.4818$ . The methyl octobromoarachidate from which the ester was prepared melted at  $228\text{--}231^\circ$ .

*Preparation of Arachidonic Acid.*—The remainder of the methyl arachidonate after the necessary analyses had been made, about 5 gm., was saponified by warming with concentrated KOH solution to which had been added an equal volume of alcohol. The soaps were dissolved in warm water and acidified with HCl. After being boiled a few minutes to clarify the oily layer, the mixture was cooled (under  $\text{CO}_2$ ) and ether added. The ether solution was separated, and the ether removed by heating under reduced pressure. (Too much heat at this stage is liable to polymerize the acid.) The acid was preserved under  $\text{CO}_2$ . Although the operations were carried out as rapidly as possible to avoid oxidation, which is especially rapid in alkali, the analytical results showed that some oxidation had taken place during the saponification. The arachidonic acid thus obtained was a light amber oil, not nearly so mobile as the ester, with a distinct fishy odor.  $n_D^{25} = 1.5563$ . Iodine number 316.2 (theory 334). The molecular weight by titration was 306.8 (theory 304). Polybromide number 80.4 (theory 210).

## DISCUSSION OF RESULTS.

The data from the analysis of the fractionated methyl esters of liver lipids are in perfect agreement with Hartley's statement that the fatty acids of these lipids contain palmitic, stearic, oleic, linolic, and arachidonic acids. The most important fact to be noted concerning these data is that the bromine content of the bromides of the several fractions is practically constant and is very close to the theoretical for methyl octobromoarachidate, and is in no case more than the theoretical. The bromides in each case were prepared from the remainder of the fraction after the other determinations had been made. Since the bromides were purified by repeated washing with ether, and not by recrystallization, one would expect to find them contain slightly lower than the theoretical amount of bromine, which is in general the result obtained. Methyl hexabromostearate, for example, would be present if linolenic acid were found in the original fatty acids. This ester is insoluble in ether and would contaminate all of the fractions, giving lower bromine analyses proportionate to the amount present. Also the comparatively low melting point of this ester bromide ( $155^{\circ}$ ) would result in some manifestation of its presence at this temperature. There was, however, no sign of sintering at  $155^{\circ}$ . Bromides from fractions (1) and (4) melted with evolution of gas at  $228-231^{\circ}$ . It is concluded, therefore, that arachidonic acid is the only highly unsaturated fatty acid present in appreciable quantities.

Two methods are available for a calculation of the amount of arachidonic acid in the fatty acids. Both are based on the polybromide number of the mixed esters. The polybromide number was found to be 6.0, or in other words, 1 gm. of ester gave 0.06 gm. of ether-insoluble bromide. A theoretical bromine content of 66.78 being assumed, this amount of bromide would contain 0.02 gm. of methyl arachidonate or 2 per cent of the total. On the other hand, it was found that methyl arachidonate gives far lower than the theoretical polybromide number, *i.e.* 77.6. On the assumption that the methyl arachidonate present in the mixed esters is the same as that prepared by reduction of the pure bromides, the original polybromide number being far too low, the

amount of methyl arachidonate present can be calculated as follows:

$$\frac{6.0}{77.6} \times 100 = 7.7 \text{ per cent.}$$

There is no way of knowing at present whether this last assumption is correct, but the writer believes that the latter method is more nearly accurate than the former.

A further series of lipids of spleen, suprarenal, thyroid, and brain is at present being studied in this laboratory with the object of finding by working with comparatively large quantities of raw material whether arachidonic acid is the only highly unsaturated acid present in these tissues.

#### SUMMARY.

1. Arachidonic acid is the only highly unsaturated fatty acid present in appreciable quantities in the lipids of pig liver. It occurs to the extent of 2.0 to 7.7 per cent of the total fatty acids, depending on the method of calculation.

2. The properties of methyl octobromoarachidate, methyl arachidonate, and arachidonic acid are described.

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## STUDIES ON THE METABOLISM OF ESKIMOS.

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It is well known that in most human subjects ketosis appears when the material metabolized becomes restricted to protein and fat. This condition is observed after several days of fasting, on diets deficient in carbohydrate, and in diabetes in consequence of the loss of ability to metabolize carbohydrate. These facts have led to the view that a certain minimum combustion of carbohydrate is necessary to avoid ketosis. The diet of the Eskimo has long been recognized as an apparent exception to this view. Being restricted almost wholly to animal tissues and containing therefore only a very small proportion of carbohydrate, such a diet might be expected to produce a ketosis. Similar diets fed to human subjects of temperate zones do so. Although the fact appears not to have been investigated, it has been assumed that Eskimos do not exhibit ketosis on their customary diets. To account for this assumed fact it has been suggested that the Eskimos must possess an adaptation to non-carbohydrate diet.

In his analysis of the ketogenic and antiketogenic factors, the balance between which seems to determine the appearance or non-appearance of ketosis, Shaffer (1) has considered the Eskimo dietary, using the data reported by Krogh (2). According to his analysis the metabolism of the foodstuffs contained in the Eskimo dietary would not be expected to cause ketosis, because the calculated antiketogenic effect of the large protein ingestion was

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† It was the interest of Dr. P. A. Shaffer in the problem of ketosis which was primarily responsible for the nature of the investigation. He contributed generously his time and knowledge of the subject in the preparation of the manuscript.

somewhat more than enough to offset the ketogenic effect of fat plus protein.

In an effort to obtain more definite information as to the carbohydrate metabolism of the Eskimos and their liability to ketosis, the writer undertook, at Dr. Shaffer's suggestion, the experiments here reported. The observations were made in July and August, 1927, upon Baffin Island Eskimos, the writer being a member of the Putnam Baffin Island Expedition.

Before presenting the results we may make a brief statement concerning the dietary habits of Eskimos. A fuller description has been written by Krogh and Krogh (2) in connection with their studies of South Greenland Eskimos at Godhavn in 1908. The statements here given apply to Greenland Polar Eskimos and to Baffin Island Eskimos, the peoples visited by the writer.

The Eskimo in his natural state eats practically only flesh. It is impossible for him to do otherwise, as plant life of a type yielding edible material does not exist. Very occasionally a bit of kelp or a few blueberries are eaten but they cannot be considered articles of diet. All animals found in the North are eaten by Eskimos but they now depend mostly on those found in the sea. Especially is this true of the Polar Eskimos, as caribou and musk-ox are practically extinct in the Thule district. The Central Eskimos can still secure both of these animals at certain seasons of the year. The seal is the animal most constantly and most universally depended upon. Not only is its flesh highly prized but the oil obtained from it furnishes heat and light. Other animals of importance are the walrus, the narwhal, the white whale, the polar bear, and the arctic hare. Dogs are eaten only in times of stress. Fish are not found to any extent where the Polar Eskimos dwell but become increasingly plentiful farther south. Everywhere water birds are found in large numbers and extensively hunted, especially eider ducks, auks, and murre. Eggs of all these birds are consumed in large numbers. Eskimos drink large amounts of water.

The preparation of Eskimo food is simple. Most meat is eaten partly boiled and the remainder raw. Frozen meat is generally eaten raw. When food of any type has been cached for some time and has become "high" or actually rotten, it is eaten raw. No salt is available.



The amount of meat eaten is very large. In times of plenty an average adult consumes 4 to 8 pounds in a day. Growing adolescents require even more. Babies are wholly breast-fed until the end of the 2nd year when meat is added to their diet. Children often nurse irregularly until the age of 4 or 6. The amount of fat eaten varies with the individual and the season of the year. In warm weather about one-seventh of the meat may be fat, in cold weather, especially when the Eskimos are traveling, one-third to one-half may be taken as fat. Krogh and Krogh have analyzed lean seal meat and found it to contain 6 to 10 per cent of fat. According to their data the average daily food partition is about 280 gm. of protein, 135 gm. of fat, and 54 gm. of carbohydrate of which the bulk is derived from the glycogen of the meat eaten.

The experiments of this report were made at Cape Dorset situated at the southwest end of Hudson Straits where a camp was established for a period of 6 weeks. The subjects utilized for the metabolism experiments were housed in a tent about 10 yards distant from the writer's, well separated from the tents of their fellow Eskimos, and were under almost constant supervision. Subjects were chosen whose physical characteristics were typical of pure blooded Eskimos. Except for short intervals they had always lived on the normal Eskimo diet. All subjects were found extremely cooperative and no difficulty was encountered in our efforts to collect desired specimens.

The main objects of the experiments were to learn whether detectable ketosis exists among Eskimos under natural dietary conditions; the extent to which ketosis develops in fasting and the rate at which it disappears on glucose ingestion; the "carbohydrate tolerance" as indicated by blood sugar curves; and to determine the respiratory metabolism during and after a ketosis-producing fast. Owing to difficulties imposed by the very limited facilities and low temperature, the respiratory data are doubtless less reliable than if obtained under laboratory conditions. But with this reservation the writer can find no reason to question the approximate correctness of the analytical results.

It may be said at once that the Eskimo on his usual dietary shows no ketosis and has high tolerance to ingested glucose. On

fasting he develops a ketosis, but only of mild degree compared to that observed with other human subjects.

*Carbohydrate Tolerance as Indicated by Blood Sugar Curves.*

With four subjects, three of whom were females, blood sugar was followed during  $3\frac{1}{2}$  and 4 hours after administration of glucose in a postabsorptive state, about 12 hours after the previous

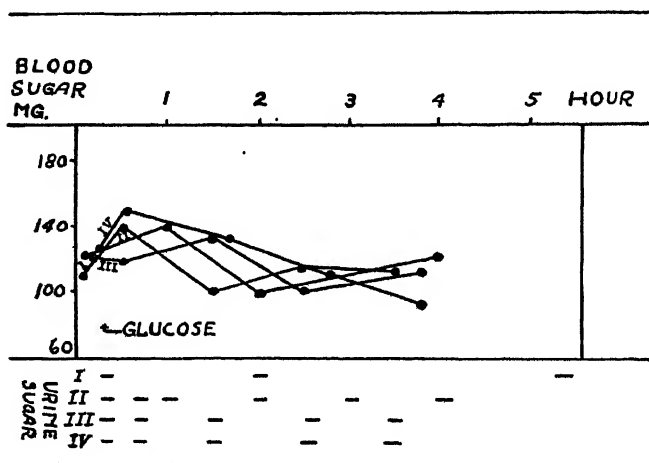


FIG. 1. Normal glucose tolerance curves. Subject I. Female, estimated age 23 yrs., weight 52 kilos; cerelese given 125 gm. Subject II. Male, estimated age 14 yrs., weight 52 kilos; cerelese given 110 gm. Subject III. Female, estimated age 13 yrs., weight 50 kilos; cerelese given 100 gm. Subject IV. Female, estimated age 12 yrs., weight 48 kilos; cerelese given 90 gm.

meal. After a preliminary blood sample was drawn, the subject drank the glucose solution, about 700 cc. containing amounts stated in legends to Figs. 1 to 3. Blood samples were drawn from an arm vein at half hour or hour intervals. In most instances urine samples were secured at the same times. The bloods were analyzed at once by the Shaffer-Hartmann method after precipitation with tungstic acid. The urines were tested qualitatively for sugar with the same copper reagent used for blood.

In order to learn the effect of fasting, the same glucose tolerance

test was performed with three subjects at the conclusion of an 82 hour fast, the glucose test dose being the first food given. The results are given in Figs. 1 to 3.

As indicated by Fig. 1, the blood sugar curves of these Eskimos, following a large dose (about 2 gm. per kilo) of glucose given 12

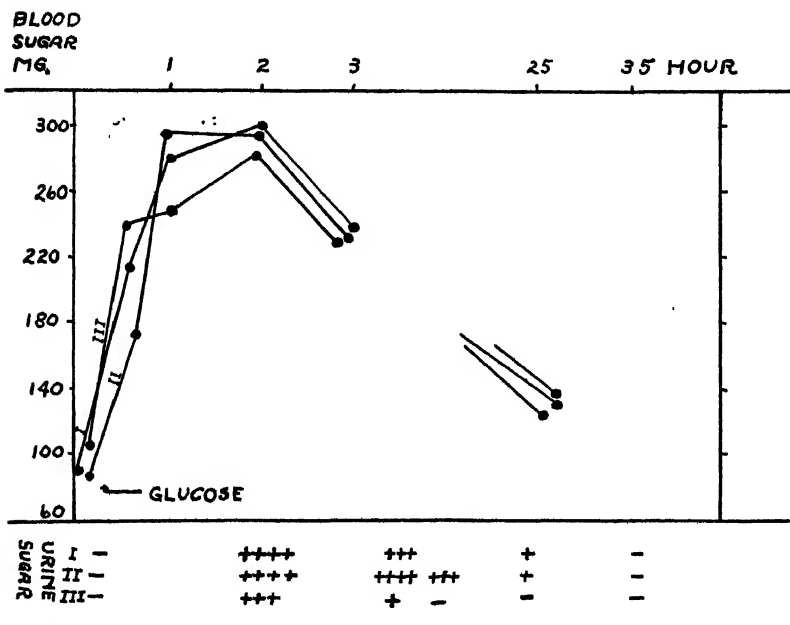


FIG. 2. Glucose tolerance curves after 82 hours of fasting. Subject I. Female, estimated age 26 yrs., weight 60 kilos; cerelose given 125 gm. Subject II. Female, estimated age 25 yrs., weight 63 kilos; cerelose given 125 gm. Subject III. Male, estimated age 28 yrs., weight 72 kilos; cerelose given 125 gm.

hours after food, show only slight rise, followed by the usual fall below the fasting value. The extent of the rise is less than commonly observed with normal subjects, and perhaps is to be interpreted as meaning exceptionally high carbohydrate tolerance. All of the urine specimens were free from sugar.

After a period of fasting, however, the behavior of the subjects

to similar doses of glucose was quite different, as shown in Fig. 2. In all three cases the blood sugar rose to maxima of 280 to 300 in 1 to 2 hours and after 3 hours was 230 to 240 mg. per cent. The first urine specimens voided 2 hours after glucose ingestion contained much sugar, and the glycosuria continued for at least 10 hours in 2 cases and for  $4\frac{1}{2}$  hours in the other. 25 hours after

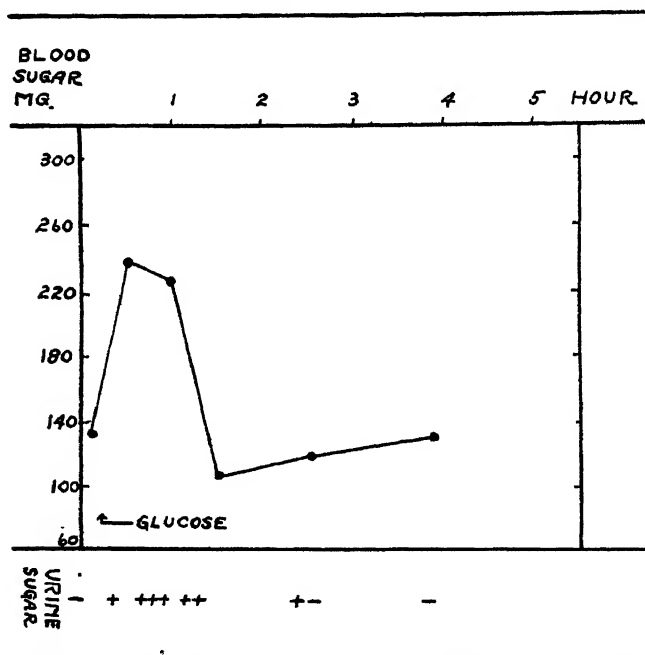


FIG. 3. Glucose tolerance curve 48 hours after Curve III, Fig. 2. Subject same as Curve III, Fig. 2.

glucose was taken the blood sugar was still somewhat higher than the fasting level.

Fig. 3 shows the blood sugar curve on Subject III after a second glucose test dose given 48 hours after the first test (Curve III, Fig. 2). During the interval he had eaten little except the 125 gm. of glucose of the first test, because his "stomach had shrunk so much during the fast that there was no room for food." In a half hour the blood sugar rose from 130 to 240 mg. and sugar appeared

in the urine. The glycosuria continued for 2.5 hours, though blood sugar dropped in 1.5 hours to below the initial value.

These results show in the first place, *that the low carbohydrate*

TABLE I.  
*Non-Protein Nitrogen of Eskimo Bloods.*

Subject.	Date.	Description of blood filtrates.	Non-protein N, mg. per 100 cc. whole blood.
I. Aleuti.	Aug. 2	Fasting blood filtrate.	30
		Mixed blood filtrates (II, III, IV).	40
		Last blood filtrate of glucose tolerance curve.	112
	3	Fasting blood filtrate.	43
II. Martha.	2	Fasting blood filtrate.	32
		Mixed blood filtrates (II, III, IV).	33
		Last filtrate glucose tolerance curve.	30
	3	Fasting blood filtrate.	38
III. Poulussi.	2	Fasting blood filtrate (I).	31
		Mixed blood filtrates (II, III, IV).	33
		Last blood filtrate of glucose tolerance curve.	29
	3	Fasting blood filtrate.	43
	4	Mixed blood filtrates of glucose tolerance curve.	36
I. (Normal curve.)		Mixed blood filtrates of glucose tolerance curve.	30
II.		Mixed blood filtrates of glucose tolerance curve.	36
III.		Mixed blood filtrates of glucose tolerance curve.	29

*content of the Eskimo diet does not result in a low carbohydrate tolerance.* This is in agreement with the observation of Greenwald and coworkers (3) that high protein diets tend to prevent the fall of tolerance which accompanies fasting or low carbohydrate,

low protein diets. The sugar formed from protein is doubtless an efficient stimulant of the pancreas or other mechanisms. As a result of fasting the high natural tolerance of the subjects was temporarily much decreased, as demonstrated by the great and prolonged rise of blood sugar and simultaneous glycosuria which resulted from glucose ingestion. But in this respect also, the Eskimo is not exceptional, for similar loss of carbohydrate tolerance from fasting has been repeatedly observed with other human subjects and with animals by Greenwald (3) and Staub (4). The phenomenon is probably to be attributed to a quiescent state of the carbohydrate mechanism (perhaps the pancreas), resulting from disuse and requiring a new stimulus to arouse it to activity. The normal height of the 12 hour fasting blood sugar of the Eskimo subjects is slightly higher than usually observed, averaging about 120 mg. per cent. This value was lowered to about 100 mg. as a result of the fast.

#### *Blood Non-Protein Nitrogen.*

The excess blood filtrates from the blood sugar determinations were brought to St. Louis and analyzed for non-protein nitrogen by distillation and Nesslerization. On arrival a slight growth of mold was evident in all of the filtrates, but the error thus introduced is probably very small. The results are given in Table I. The last blood filtrate of the glucose tolerance test for Subject I yielded 112 mg. of non-protein nitrogen. This analysis was repeated with the same result and is perhaps due to faulty precipitation of the blood proteins. The other figures fall within the usual normal range and indicate no retention of nitrogenous products in the blood from the habitual high protein diets.

#### *Ketosis during Fasting.*

Three of the subjects were fasted for 3 days, during which time the urine was collected, measured, and the specimens tested qualitatively for acetoacetic acid, both with ferric chloride and nitroprusside,  $\text{NH}_4\text{SO}_4$ , and  $\text{NH}_4\text{OH}$  (Rothera's test).

About noon and in the evening of each day acetone was tested for in the breath of each subject. The air was exhaled for 2 or 3 minutes through a glass tube dipping into cold distilled water,

after which the addition of Scott-Wilson reagent caused opalescence if acetone were present. This test is very delicate.

Samples of the urine specimens were bottled with toluene and brought to St. Louis, where they were analyzed for acetone, diacetic, and  $\beta$ -hydroxybutyric acid by the Shaffer-Marriott method,<sup>1</sup> and for total nitrogen by the Kjeldahl method. Acetoacetic acid had disappeared from the urines on arrival in St. Louis, about 3 months after the experiments were conducted. There is, however no reason to doubt the reliability of the total acetone body deter-

TABLE II.  
*Qualitative and Quantitative Urine Analyses.*  
*Qualitative Test for Acetone in Breath.*

Subject.	Day of fast.	24 hour urine volume.	Total N.	Total acetone bodies in urine as $\beta$ -hydroxybutyric acid.	Qualitative tests for acetone bodies in:	
					Breath.	Urine.
I. Aleuti.	1	cc.	gm.	gm.	0	0
	2	1400	18.90	0.105	+	+
	3	2130	14.60	0.180	++	++
II. Martha.	1	1520	14.74	0.300		
	1	1380	24.60	0.097	0	0
	2	1400	13.50	0.320	++	++
III. Poulussi.	3	1890	15.40	1.230	+++	+++
	1	1420	21.20	0.125	0	0
	2	1250	13.60	0.132	+	+
	3	1460	15.55	0.160	+	+

minations. The results obtained for the total acetone bodies show much less striking increase with the progress of the fast than indicated by the qualitative tests made at the time. In view of the fact that the total amount of acetone plus acetoacetic and  $\beta$ -hydroxybutyric acids in specimens of well preserved diabetic urines does not change significantly in periods of a week or two, we have assumed that no loss of total acetone occurred in these urines and that the analytical results represent the amounts originally present. As a check a diabetic urine under toluene was analyzed for total acetone bodies before and after 17 days of incubation at

<sup>1</sup> I am indebted to Dr. P. A. Shaffer for these analyses.

38°. There was no loss of total acetone bodies. Such incubation was considered more likely to produce decomposition than several months of preservation at ordinary temperature. The results of urine analyses and of the qualitative tests made at the time are shown in Tables II and III.

The qualitative tests of urine and breath show that the Eskimos did develop distinct ketosis on the 2nd day of fasting, which increased somewhat on the 3rd day. The tests were less marked with Subject III than with the others. Subject II, who showed the highest ketosis, was a nursing mother who nursed her year-old baby throughout the fast. The formation of lactose was presumably an additional drain on the glycogen reserves. Although the results of quantitative determinations in urine also rise during the fast,

TABLE III.

*Rate of Disappearance of Acetone from Breath and Acetoacetic Acid from Urine after Glucose and Resumption of Food.*

Hrs. after breaking fast with 125 gm. glucose.	Subject I.		Subject II.		Subject III.	
	Urine.	Breath.	Urine.	Breath.	Urine.	Breath.
2	+	+	+	+	±	+ (Slight.)
4.5	+	+	+	+	±	+
6.5	-	+	+	+	-	+
10.0	-	-	±	+	-	-
25.0	-	-	-	-	±	+ (Slight.)
35.0	-	-	-	-	-	-

the total amounts are surprisingly small, the highest on the 3rd fast day of Subject II being equivalent to only 1.2 gm. of  $\beta$ -hydroxy-butyric acid. The maximum excretion by the others was only 0.3 gm. and 0.160 gm.

The amounts of acetone bodies excreted by different subjects during fasting vary greatly. Some may hesitate to grant that the small amounts excreted by these Eskimos indicate a relative non-susceptibility to ketosis. It is unfortunate that the fasts were not prolonged; but the positive qualitative tests in urine and breath on the 2nd day becoming more pronounced on the 3rd day, seemed to answer the main question whether a fasting ketosis would appear, and it was only when the urines were analyzed some months later that the slowness of the degree of ketosis was



appreciated. As examples of variation in the amounts of total acetone bodies excreted by other human subjects during short fasts the data in Table IV may be cited.

As a rule most subjects show a ketosis of a gm. or more of total acetone on the 3rd fast day, but it will be noted that after an interval following one or two fasting periods, the obese subjects of Folin-Denis showed slight or no ketosis on the 3rd fast day. Marked ketosis did appear in these subjects on the 4th or 5th fast days. However, the Eskimo subjects had not fasted previous to our tests.

Why the ketosis observed with the Eskimo subjects is so mild, it is perhaps impossible to decide with the evidence available.

TABLE IV.

		Excreted on 3rd fast day. Total acetone bodies as $\beta$ -hydroxybutyric acid.
		gm.
Benedict (5).	Subject L., weight 60.1 kilos.	2.1
Folin-Denis (6).	Mrs. M. Obese, weight 108 kilos.	
	First fast.	20.1
	Second "	1.4
	Third "	0.55
Folin-Denis (6).	Mrs. B. Obese, weight 178 kilos.	
	First fast.	1.95
	Second "	0.0
	Third "	0.0

We may suppose either that marked ketosis was prevented by a continued combustion of small amounts of glycogen, thus providing a ketogenic balance as postulated in Shaffer's analysis, or we may assume that little or no glycogen was available and that the Eskimo actually does possess an adaptation permitting complete combustion of fat without any intervention of carbohydrate and in this respect differs from other human subjects. Before considering these two possibilities we shall present the data on respiratory metabolism, which were obtained in the hope of deciding between these two interpretations.

*Respiratory Metabolism.*

Daily during the fasts, determinations of the respiratory metabolism were made. The expired air was collected in 5 minute periods in a Douglas bag, after which the volume was measured by

TABLE V.  
*Respiratory Exchange during Fasting.*

Day of fast.	Body temperature.	Pulse rate.	Respiratory rate.	Cc. O <sub>2</sub> consumed per min.	r.q.	Calories per hr.	Calories per sq. m. body surface per hr.	Du Bois standard.
Subject I. Female, age 26 yrs., weight 60 kilos, height 153 cm.; surface area 1.57 sq.m.								
1	98.0	76	20	224	0.982	67.80	50.7	37
2	98.2	71	10	303	0.806	87.60		
3*	98.0	73	18	282	0.640	79.20		
3.5	Basal metabolism not determined.				0.600			
Subject II. Nursing female, age 25 yrs., weight 63 kilos, height 159 cm.; surface area 1.65 sq.m.								
1	98.2	70	20	314	0.813	91.20	49.6	37
2	97.2	67	16	314	0.654	87.00		
3*	97.2	72	16	291	0.608	80.40		
3.5	Basal metabolism not determined.				0.454			
Subject III. Male, age 28 yrs., weight 72 kilos, height 160 cm.; surface area 1.75 sq.m.								
1	98.1	72	24	270	0.81	78.00	51.4	39.9
2	96.3	62	17	214	0.865	62.40		
3*	98.0	57	18	320	0.714	90.00		
3.5	Basal metabolism not determined.				0.676			

\* Day on which checks were made.

water displacement in a glass cylinder, and samples analyzed for O<sub>2</sub> and CO<sub>2</sub> in a Henderson-Haldane gas burette. The subjects, dressed in their usual clothing, rested on a cot for 30 minutes before and during each respiration period. The temperature of

the tent varied from 10 to 14°. Body temperature (mouth), pulse, and respiration rates were recorded. The results of the respiratory exchange are given in Table V. The data are not as regular and consistent as would be desired, and may possibly be open to question. The conditions under which the work was done were not favorable. On the other hand, care was taken to detect and avoid errors of manipulation and analysis. The bag was tested for leaks. Atmospheric air and the writer's own alveolar air were repeatedly analyzed with satisfactory results as a check on the gas analysis. Duplicate determinations were possible only on the 3rd fast day because of a lack of assistants.

The results call for comment on several points. In each of the three subjects the first determinations, made in the postabsorptive state about 10 or 12 hours after the last food, give normal R.Q., but the total metabolism is much higher than the standards for subjects in the temperate zone. It is suggestive that the resting metabolism of these subjects living near the arctic is about 33 per cent greater than the values for the temperate zone; while according to a report by de Almeida (7) the metabolism is 24 per cent less for subjects in tropical regions. The total metabolism showed no tendency to fall except in Subject II, and in Subject III on the 2nd day when the drop from 78 to 62 calories per hour may perhaps be associated with the low body temperature at the time. The absence of a consistent fall of metabolism with fasting suggests that the high values are not merely the temporary immediate effects of the customary high protein ingestion. It would appear that the metabolism is adjusted to a higher level, an inference which may be related to the apparent high vascularity of these subjects. On the 3rd day of fast with each subject and on the 2nd with Subject II (this one developed the most marked ketosis), the R.Q. was at or below the values indicative of complete cessation of carbohydrate combustion. It will be noted that values of 0.64, 0.60, and 0.71 were observed on this day. With only one series of experiments one hesitates to state too positively that these represent true metabolic quotients. They are, however, the same on all subjects. No analytical defect could be detected and experimentally they are believed by the writer to be correct. They certainly leave no ground for supposing that the subjects were oxidizing carbohydrate, and some other explanation is necessary to account for the small amount of ketosis.

The conventional interpretation of such low quotients is of course that of carbohydrate formation (and storage) from fat. The acceptability of such interpretation will depend upon whether the reader favors or rejects the existence of this transformation. No other evidence is at hand to decide this controversial question; but if the hypothetical carbohydrate formed from fat, as suggested by the low R.Q., were stored as glycogen (only a part of it could have been burned), it seems surprising that the subjects should react as they did to glucose ingestion. The marked hyperglycemia and glycosuria certainly indicate that the subjects had a temporarily decreased power to store or oxidize ingested glucose.

In relation to the problem of accounting for the slightrness of the ketosis found in the Eskimo subjects, it is therefore apparent that they, like the obese subjects referred to above, require less carbohydrate to burn their fat than normal white subjects. Children on the other hand, as shown by Wilson *et al.* (8) require a larger proportion of carbohydrate to permit the complete combustion of fat. All these data are consistent with the hypothesis that there are two mechanisms possessed by the animal organism for the combustion of fat, one of which involves the coincident combustion of carbohydrate. The varying ketogenic-antiketogenic ratio might then be a function of the relative effectiveness of these two biological mechanisms, with the further possibility that adaptation can be brought about to a low carbohydrate diet by an increase in the effectiveness of the direct mechanism for the burning of fat.

#### SUMMARY.

A brief account of the diet of Polar and Baffin Island Eskimos is given.

By means of glucose tolerance curves it is shown that these people have a high tolerance for carbohydrate. Following a period of fasting this tolerance is markedly decreased.

The non-protein nitrogen of the blood of Eskimos is similar to that of other races. The results indicate no retention of nitrogenous products in the blood from the habitual high protein diets.

Eskimos show a remarkable power to oxidize fats completely, as evidenced by the small amount of acetone bodies excreted in the urine in fasting.

The basal metabolism of Eskimos is considerably higher than that of persons living in temperate zones.

During fasting the respiratory quotient falls to a level which may be interpreted as indicating a conversion of fat into carbohydrate.

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## BLOOD UROBILIN.

### THE UROBILIN CONTENT OF NORMAL HUMAN BLOOD. DESCRIPTION OF A METHOD.

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Normal urobilin content of human blood has not been described. Present knowledge of the normal metabolism of urobilin deals indirectly with that phase which concerns the blood. The physiology of urobilin in animals also deals indirectly with the subject of blood urobilin. Elman and McMaster (1), in their experimental studies in urobilin physiology, had no method for blood urobilin.

The exact nature of urobilin is, perhaps, not known; but with the available methods for bile, feces, and urine, a large amount of fact about urobilin has accumulated and there is no serious discord in interpretation. Practically all urobilin studies have been done with one or the other of two methods; namely, the Schlesinger fluorescence method and the Ehrlich aldehyde test. The Ehrlich test is generally found to be not very sensitive, incapable of detecting urobilin in the blood of normal man or animal, and, furthermore, subject to the criticism that it reacts to tryptophane (Marechal (2)). The Schlesinger test is known to be very sensitive (von Fisher (3), 0.0048 mg. per 100 cc.) and highly specific.

Garrod and Hopkins (4), Biffi (5), Meyer-Betz (6), Obermayer (7), Grigaut (8), von Fisher (3), and Palmer (9) have worked with urobilin and accepted this test as specific, but no one has uniformly found the test to be positive in human blood, although urobilin is always present in urine.

A few methods for blood urobilin based on the Ehrlich test or Schlesinger test have been reported, the authors finding urobilin in abnormal conditions such as pneumonia, nephritis, or cirrhosis of the liver, where, for various reasons, a high concentration of urobilin was present. Brule and Weiss-

man (10), Grigaut (8), Weltmann and Löwenstein (11), Eppinger and Ranzi (12), Hildebrandt (13), Rodillon (14), Winternitz (15), Blankenhorn (16), and Royer (17) comprise the entire literature on the subject. Schlesinger's test has been greatly improved by the work of Marcussen and Hansen (18), who were particularly concerned with the nature of its fluorescence and described the optimum conditions for fluorescence with urobilin and zinc salts. The work of Elman and McMaster (1), who made the Schlesinger test quantitative by developing a standard, together with that of Marcussen and Hansen, has made possible the work reported here.

The method we describe is the application of the fluorescence test to blood serum, modified to make it more sensitive by refinements in two main principles; namely, to provide absolute clear supernatant solutions in which to develop fluorescence, and to examine these solutions in a dark room with an intense beam of light. By these refinements in technique it has been possible to detect fluorescence in practically every normal human blood. This fluorescence has been measured quantitatively by using the Elman and McMaster (1) standard. The details of the technique are as follows: Blood is obtained by venepuncture and allowed to clot for at least 12 hours. Oxalate and fluoride plasma have been used, but clearing seems more difficult than when serum is used. The clot is freed from the walls of the tube and centrifuged to give a clear serum without hemolysis. 2 or 3 cc. of clear serum are accurately pipetted off and placed in a special tube on about 0.5 gm. of finely powdered c.p. zinc acetate (the zinc is an excess and need not be measured carefully). This is shaken and given time to mix so that acid coagulation of the serum results. In a minute or two, 3 volumes of absolute alcohol are added and the specimen well mixed. The tube is then corked and placed in the ice box for 24 hours. After 24 hours the specimen is centrifuged, if clear, and the supernatant liquid is poured off into a similar tube and the urobilin estimated by the method described below. Certain precautions are observed to guarantee perfectly clear supernatant liquids; *viz.*, uniform mixing of specimen so that a good flocculent coagulation is formed; specimens are not centrifuged until a good coagulation with clumping or flocculation has resulted. It has been found best to pour off the supernatant liquid carefully without carrying over any of the sediment, since it is not necessary to pour off all of the supernatant liquid. Absolute alcohol must be used because all other varieties of alcohol,



regardless of percentage or purity, contain a blue fluorescent substance which interferes with readings and is got rid of with great difficulty. It is necessary to keep the specimens in the ice box and to centrifuge immediately on removing from the ice box, so that specimens can be read at a slightly higher temperature than the temperature at which they were cleared. Specimens must be corked to prevent evaporation. Great care must be taken in the preparation of all glassware in order to prevent any turbidity of the supernatant liquid. It is frequently unnecessary to pour it off, and specimens can sometimes be read in the original tube. This is desirable because pouring into a new tube frequently causes turbidity for some strange reason—possibly minute particles of dust, perhaps slight changes in pH due to the action of glass. In general, the less specimens are handled the more likely they are to remain clear. Filtering of specimens is never employed because of the turbidity that arises from various causes; *i.e.*, dust on the filter paper and lint from even the hardest of papers. Furthermore, repeated filtering has been found entirely to remove the fluorescent substance, possibly by adsorption to the paper.

If turbidity persists regardless of all precautions, specimens can occasionally be cleared by adding as much protein as is contained in a platinum loop of the serum originally employed. This minute addition introduces a negligible error and may turn the trick.

Thick-walled, culture tubes,  $\frac{1}{2} \times 4$  inches, are carefully cleaned in acid cleaner and many rinsings of distilled water, dried in a drying oven, and then carefully protected from dust while in storage. These tubes are used for no other purpose and subsequent cleaning is done with Dutch Cleanser and water. Tubes are "banded" about the top so as to cover the entire lip with black paint. For this we use Service Seal black paint (sold by John A. Steen, Varnish Company, Chicago). All tubes must be carefully matched as to size, thickness of wall, and color of glass.

Comparison with the standard is made under carefully controlled circumstances, aimed to detect the most minute traces of fluorescence. The same conditions which detect minute traces of fluorescence also detect minute turbidity, or particulate matter of any origin which by causing a Tyndall phenomenon obscures a reading.

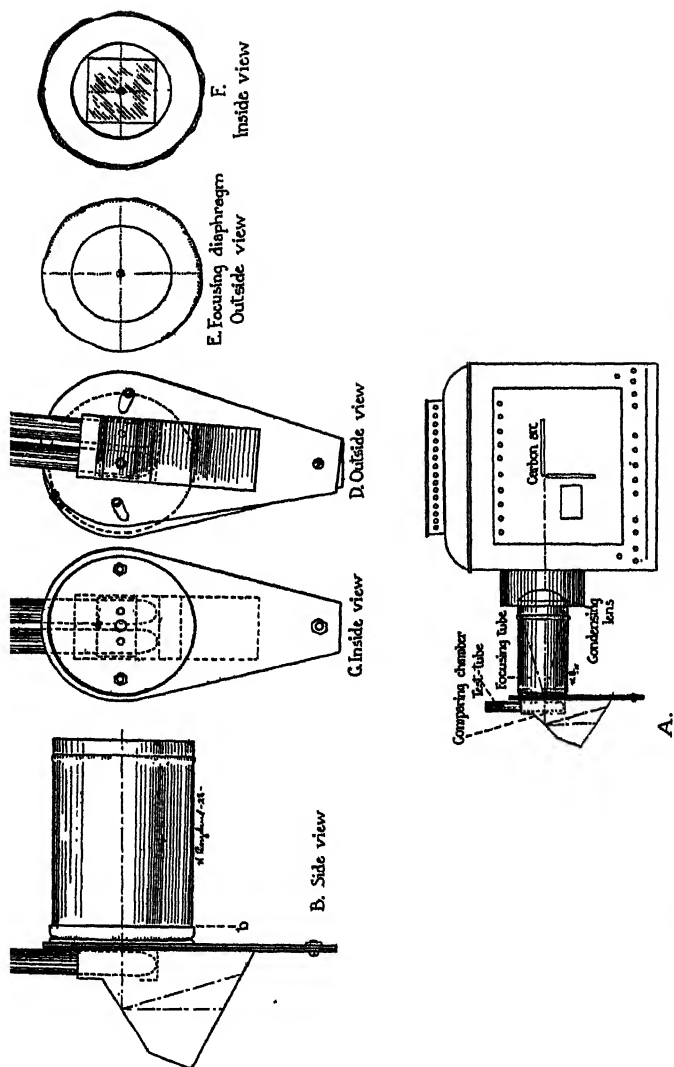


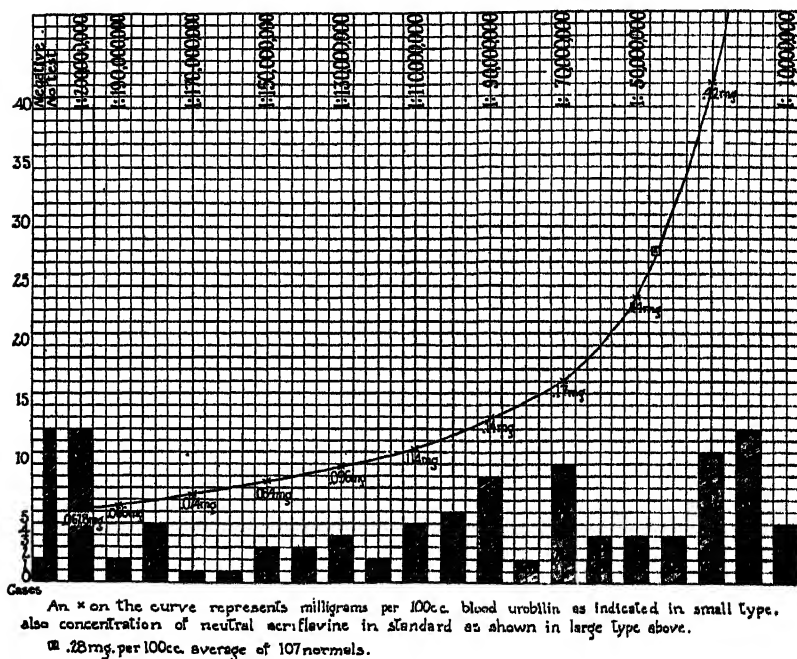
FIG. 1. A, side view of the entire apparatus; *viz.*, lamp, housing, condenser, focusing tube, and comparing chamber. B, focusing tube and comparing chamber enlarged. C and D, details of comparing chamber, showing arrangement permitting the operator to examine a pair of tubes in a single beam of light. E and F, details of focusing diaphragm to be used in adjusting the single beam of light prior to making a comparison. This diaphragm and the comparing chamber are interchangeable on the end of the focusing

An arc lamp, model D, baloptican (made by Bausch and Lomb Optical Company, Rochester, New York) is used as source of the light—this being a carbon arc in a light, tight housing and with condensing lens to bring the light to a sharp focus. At this focus, in a box of special design, two tubes can be examined by being rapidly alternately placed in the path of the beam of light as illustrated (Fig. 1). Upon working in a very dark room with this intense light, minute traces of fluorescence can be detected and estimated quantitatively by direct comparison with the standard.

The standard used is a dilute aqueous solution of neutral acriflavine (Boots Pure Drug Company) as described by Elman and McMaster (1). A parent solution is made of 0.1 gm. in 100 cc. of double-distilled water, this making a dilution of 1:1000. The parent solution keeps for 6 months if stored in an ice box. From this parent solution a stock solution is made once a week by adding 1 cc. to 99 cc. of double-distilled water. This makes a dilution of 1:100,000. The standard actually employed for the reading is made from the stock solution each day and should be used at once, because a marked loss of fluorescence can be noted in several hours at room temperature. The standard as used is a series of ten tubes, the strongest of which is a solution of 1 part in 10,000,000, and the weakest a solution of 1 part in 200,000,000. The intermediate tubes in the series are respectively, 30,000,000, 50,000,000, 70,000,000, 90,000,000, 110,000,000, 130,000,000, 150,000,000, and 170,000,000. This series was found by many trials to be as long and as regular as could be used; that is to say, that any further dilutions of the dye could no longer be seen at all, and any greater concentration, stronger than 1:10,000,000, could not be recognized as being more green. Furthermore, when a greater number of tubes was used in the series (that is more steps) there was confusion because differences of color were too slight to be recognized as steps. As a check on the standard, each series was proved by carrying out the dilution to the disappearing point and each series arranged in proper order without any reference to the labels—that is to say, the standard was considered correct when any further diluting beyond 1:200,000,000 dispelled all green color, and when there was enough color difference in each tube in the series so that each could be arranged

in proper order as to intensity of green color. In working with this standard, as well as the unknowns to be measured, it must be borne in mind that colors seen as a result of fluorescence do not behave as do colors of ordinary colored solutions. We are relying on the work of Elman and McMaster (1) to establish the correctness of the method as well as the value of the standard. It must be noted here that since publishing their method, these authors

Summary: 128 Determinations of Urobilin in Human Blood.



can be noted that in the greater dilutions, that is in all but the three strongest solutions, the strength of color is virtually a straight line function of the concentration of the dye. Nearly all specimens were diluted so as to be read on this part of the curve. In actual operation the method must necessarily be modified in order to bring the unknowns within the scope of the standard, because the standard cannot be made stronger. In order to accomplish these dilutions and not produce cloudiness in the specimens, the dilutions must be made before the entire precipitation of protein is accomplished. This somewhat difficult task must be anticipated and determined by the appearance of the specimen after the first precipitation. Inspection of the specimen is then made and the intensity of fluorescence estimated. If fluorescence is very intense further dilution must be made in order to bring the final product within the range of the standard. It has not been feasible to add alcohol to specimens after the final clearing, because it is impossible to add alcohol without either producing turbidity or else destroying the fluorescence by too great dilution of the zinc factor. With care and experience every specimen can be brought within the range of the standard and read. All normal blood specimens fall well within the lower dilutions.

All specimens were read after 24 hours standing in the ice box to precipitate before clearing in the centrifuge. The first readings were recorded and the specimens put back into the ice box unaltered, to allow further development of fluorescence, and to be read again the 2nd day and again on a 3rd day. The highest reading was taken as final. It has been found usually on the 3rd day.

Fig. 2 is a summary of 128 determinations of urobilin on human blood presumably normal. Of the 128 specimens but two were negative. In twelve instances no test could be made; this on account of inability to clear specimens. With greater experience, it is probable that no specimen will be found negative, and with good technique there should be very few with which no test can be made. The average of our series gives 0.28 mg. per 100 cc. This average may be slightly greater than is justifiable, due to including a few abnormal specimens. Blood for these tests was taken from unselected patients in a general medical clinic, where Wassermann tests were made. Blood was taken from no indi-

vidual with any definite abnormality except occasionally a positive Wassermann, and such hypothetical disorder as may result from the administration of salvarsan.

Numerous pathological specimens were measured which go as high as 33 mg. per 100 cc.—the highest values being in patients with nephritis, malaria, pneumonia, and tuberculosis with fever; and one patient with complete obstruction of the gall duct and with complete intestinal acholia was found to be clearly negative. This is in harmony with current ideas of urobilin metabolism.

Since this series was described, the technique has been simplified by use of a smaller and simpler series for the standard as follows:

*Parent Solution.*—1 gm. of acriflavine in 1000 cc. of distilled water.

*Stock Solution.*—1 cc. of parent solution in 99 cc. of distilled water. To be made up weekly.

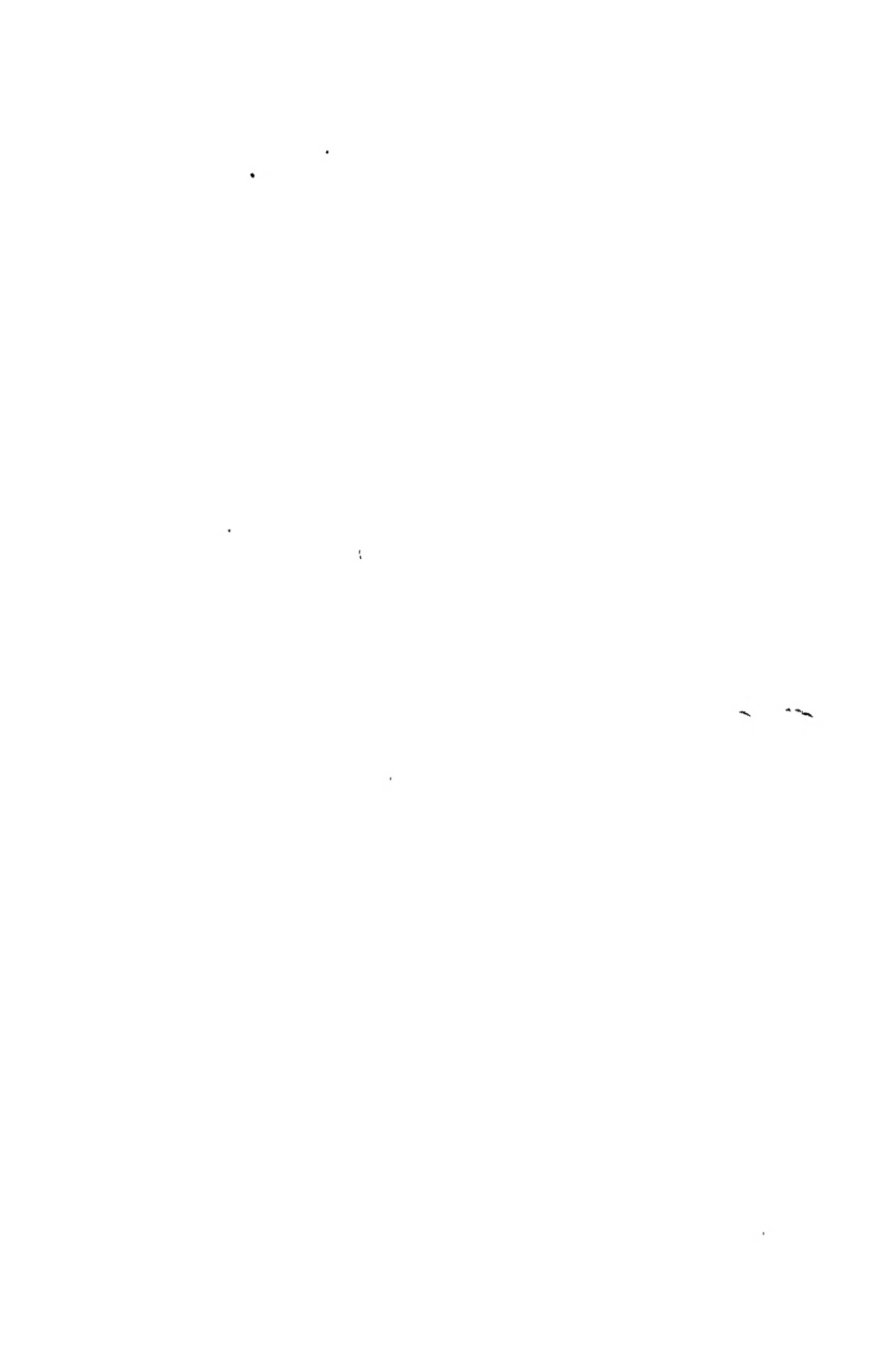
*Standard.*—Make a 1:100 dilution of the stock solution, which is Tube 1 in the series, being a 1:10,000,000 solution of the dye. The remaining five tubes of the series are made from Tube 1 by use of a graduated 1 cc. pipette for the solution and a graduated 10 cc. pipette for the water, worked into 10 cc. tubes, and with dilutions as follows: 1:3, 1:5, 1:7, 1:10, and 1:20. The final series of six tubes must contain about 3 cc. so that all solutions and unknowns are of equal depth when readings are made. From our computation, Elman and McMaster values being used and allowance made for the 1:4 dilution of the unknown, each tube of the standard has the following value in mg. of urobilin per 100 cc. of blood.

Tube 1 (1:10,000,000)	equals	0.315	mg. per 100 cc.
" 2 (1:30,000,000)	"	0.105	" " 100 "
" 3 (1:50,000,000)	"	0.06	" " 100 "
" 4 (1:70,000,000)	"	0.0425	" " 100 "
" 5 (1:100,000,000)	"	0.0315	" " 100 "
" 6 (1:200,000,000)	"	0.0157	" " 100 "

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## THE INTESTINAL pH IN EXPERIMENTAL RICKETS.

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The unfavorable effect of alkalization of the medium on absorption of calcium from the intestinal tract has been demonstrated by several investigators. Irving and Ferguson (1) found better absorption from buffered calcium chloride solutions at pH 3 than at pH 7 or higher. Increasing the alkalinity of milk has been shown to interfere with calcium absorption (2, 3), while the addition of hydrochloric acid (4) or ammonium chloride (5) seems to have the opposite effect.

By substituting for the calcium lactate in a rickets-producing diet of the high Ca-low P type, an equivalent amount of calcium chloride, which is acidogenic, Zucker, Johnson, and Barnett (6) were able to produce only a minimal degree of rickets. The addition of 2 per cent ammonium chloride to the basal diet completely inhibited the development of this condition. On the other hand, an apparently normal diet was made rachitogenic by the addition of 2 per cent sodium carbonate. These experiments were offered in support of the opinion that a "factor which resides in the individual and not in the diet" (*viz.*, the intestinal hydrogen ion concentration) might explain the occurrence of rickets in infants.

Abrahamson and Miller (7) and Grayzel and Miller (8), working on rats and dogs respectively, determined the pH at different levels of the intestinal tract following both normal and rachitogenic diets. The latter produced higher alkalinity all along the tract, but when these diets were supplemented with antirachitic measures normal reactions were found. These studies were interpreted as demonstrating that diminished solubility of calcium salts in an alkaline medium prevented their complete absorption and resulted in rickets.

Increased fecal alkalinity in rickets has been observed by other investigators, notably Jephcott and Bacharach (9), Redman, Willimott, and Wokes (10), and Yoder (11). Following an original suggestion of Zucker and Matzner (12), Jephcott and Bacharach have applied the pH change in rickets as a test for vitamin D, which has more recently been placed on a quantitative basis (13).

The present experiments were undertaken before some of the above reports were published. It was desired to examine the feasibility of the fecal pH test as a measure of vitamin D potency,

and also to study the relation of the hydrogen ion changes to the etiology of rickets.

#### EXPERIMENTAL.

Albino rats were fed the Steenbock and Black rickets-producing diet, Ration 2965 (14), modified by additions of 2 per cent ammonium chloride (Diet 110), 2 per cent sodium carbonate (Diet 111), and 4.2 per cent sodium lactate (Diet 112). The basal diet was designated Diet 108. The proportion of sodium lactate was determined by its equivalence to 2 per cent sodium carbonate on the basis of sodium content, although it was expected that the lactate would be even more alkaligenic owing to the base-binding power of the  $\text{CO}_2$  which would be yielded upon oxidation.

Semiweekly determinations of the pH of the feces were made, the feces having been collected from clean blotting paper which had been placed in the cage pans the same day. If natural evacuation failed to occur within a few hours, specimens were obtained by digital expression from the rectum. Growth and food consumption records were also made twice weekly. The condition of the leg bones was followed radiographically, at least once each week. Ultra-violet radiation was provided by a Hanovia quartz mercury vapor lamp operated at 60 to 65 volts at a distance of 30 inches from the animals. Cod liver oil was fed apart from the diet, diluted in mineral oil. In certain of the preventive experiments, the particular oil used proved to be inadequate at 6 mg. daily, and the dose was therefore increased first to 8 mg. then to 10 mg., as indicated on the curves.

pH determinations were conducted colorimetrically on the filtrate from a homogeneous suspension of the fecal material in distilled water, the Walpole compensation principle being used. Standards ranging from pH 6.2 to 8.2 were prepared from  $\text{m}/15$  phosphate buffer solutions in intervals of 0.2 pH. Since the purpose of these estimations was to compare relative values, *e.g.* before and after the application of antirachitic measures, absolute pH values were not expected. Justification of the colorimetric method may be found in the work of Grayzel and Miller (8) in which the average error was estimated to be  $-0.2$  pH, while comparison of the colorimetric with the electrometric method "justifies reliance on comparative results as obtained colorimetrically."

Preliminary experiments showed that since different specimens varied in their buffer capacity, the use of a definite degree of dilution would have little significance. It was observed however, that as long as the suspensions retained their turbidity they were sufficiently buffered to prevent significant variation from the original pH value. The filtrates themselves could be still further diluted without altering the pH value.

Animals were killed by asphyxiation with illuminating gas. At autopsy the entire intestinal tract was removed and divided into four sections, the upper and lower ileum, the cecum, and the colon. The contents of these portions were gently expressed and the pH determined. Examination for gross pathology was also made, chief emphasis being laid on the condition of the ribs and spine, beading, enlargement, curvature, and fractures being particularly noted.

### *Results.*

Typical curves representing fecal pH are shown according to the diets fed, Fig. 1 representing those rats receiving the unmodified basal diet. Figs. 2 to 4 represent the ammonium chloride, sodium carbonate, and sodium lactate modifications respectively.

The fecal pH curves are usually irregular. Even where a definite tendency in one direction or another obtains, there are observed occasional marked divergences from the general trend. In some cases a well marked rhythmicity was observed, usually when the rat was continued on the same dietary régime for a period of time (see Rat 2087, Fig. 1, Rats 2080, 2066, Fig. 2, Rat 2082, Fig. 3, *etc.*). Furthermore there was occasionally noted a parallelism in the fluctuations of fecal pH in two rats from the same litter, although the actual pH levels might vary (see Rats 2072, 2073, Fig. 1, Rats 2078, 2079, Fig. 4). Similar "waves" in calcium metabolism and in fecal reaction have been reported by Willimott and Wokes (15).

No definite pH zone could be established which might function as a border zone between the normal and the rachitic fecal reaction. While the general rule was that high pH values prevail under rachitic conditions and low values follow curative or preventive measures, exceptions to this rule were often noted. For

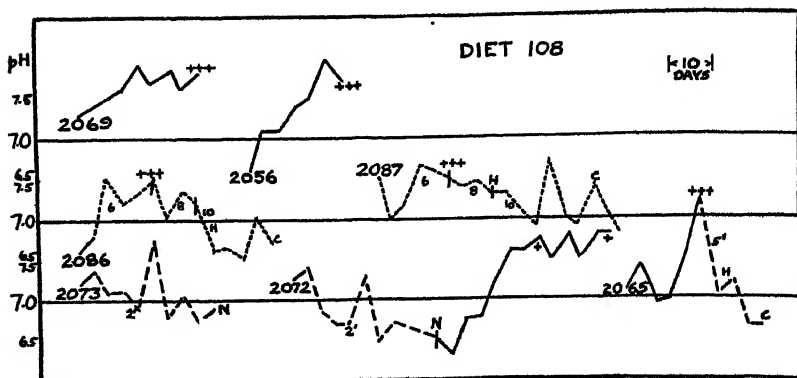


FIG. 1. Fecal pH curves of rats receiving Diet 108 (Steenbock and Black Ration 2965) alone (solid line), or supplemented with cod liver oil (mg. indicated) (dash line), or by daily exposure to ultra-violet radiation (minutes indicated) (broken line). N = normal tibia; H = beginning healing; C = cured; +++ = severe rickets.

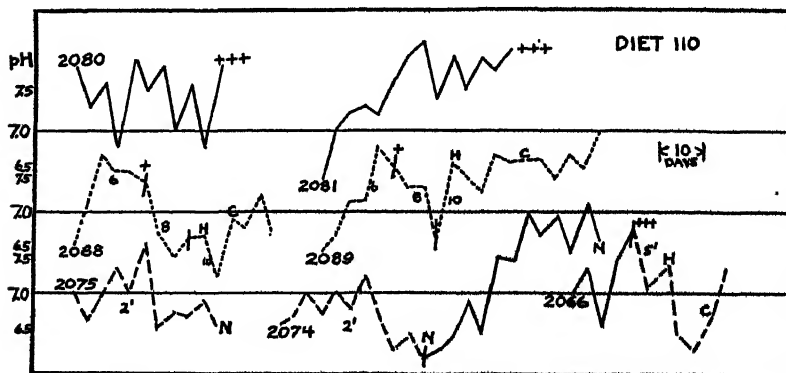


FIG. 2. Fecal pH curves of rats receiving Diet 110 (containing 2 per cent ammonium chloride) alone (solid line), or supplemented with cod liver oil (mg. indicated) (dash line), or by daily exposure to ultra-violet radiation (minutes indicated) (broken line).

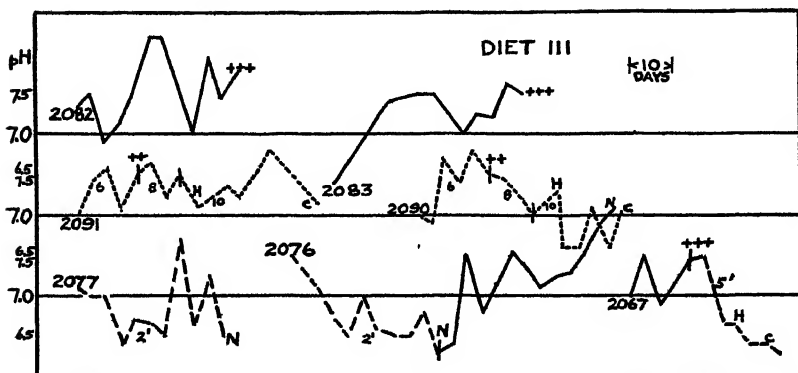


FIG. 3. Fecal pH curves of rats receiving Diet 111 (containing 2 per cent sodium carbonate) alone (solid line), or supplemented with cod liver oil (mg. indicated) (dash line), or by daily exposure to ultra-violet radiation (minutes indicated) (broken line).

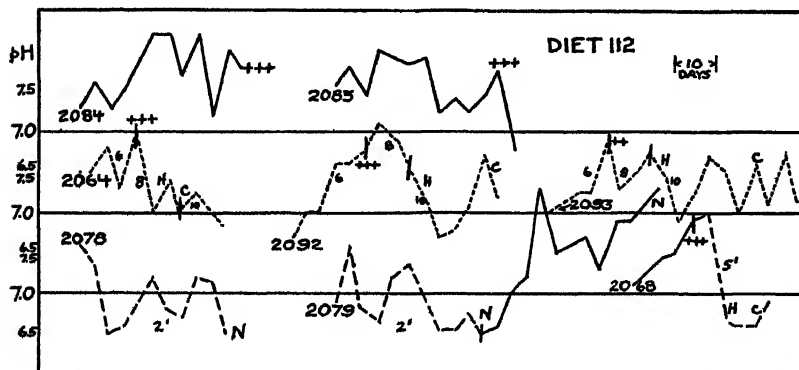


FIG. 4. Fecal pH curves of rats receiving Diet 112 (containing 4.2 per cent sodium lactate) alone (solid line), or supplemented with cod liver oil (mg. indicated) (dash line), or by daily exposure to ultra-violet radiation (minutes indicated) (broken line).

example increased fecal alkalinity was observed in Rat 2087 (Fig. 1) and Rat 2093 (Fig. 4) during periods when these animals were practically cured of rickets, whereas drops in pH were observed in Rat 2080 (Fig. 2), and Rats 2082 and 2083 (Fig. 3)

while these animals were severely rachitic. The calcification attendant upon the period of inanition preceding death was also associated with a drop in pH, but this is not included in the above comment.

The pH value of feces therefore apparently lacks specificity as an index to a rachitic condition. It would seem that the change in direction of the fecal pH curve may accompany the change in diet but may not necessarily be related to altered calcification. This is illustrated by the curves for Rat 2074 (Fig. 2) and Rat 2079 (Fig. 4), showing the rise in fecal pH following the cessation of daily exposure to ultra-violet light, without the appearance of decalcified areas in the tibial metaphyses. The removal of the protection afforded by ultra-violet radiation should ultimately result in rickets (see curve for Rat 2072, Fig. 1), although it appeared that the vitamin D reserve thus formed was not sufficient to offset the deflection of the fecal pH values toward alkalinity.

No correlation was observed by Redman (16) between the degree of rickets in children and their fecal pH values, the latter having shown wide fluctuations in each case.

Neither the acidogenic salt ammonium chloride, nor the alkaligenic salts, sodium carbonate or lactate, when added to the diet, appeared to affect the rickets-producing property of the basal diet in the direction of either inhibition or promotion. In all cases, rickets of about the same degree of severity was produced in approximately 3 weeks. The ammonium chloride diet failed to induce recalcification when fed to rats made rachitic by the basal ration. Animals receiving the alkaline diets exhibited no observable resistance to cure by cod liver oil or exposure to ultra-violet radiation.

Moreover the curves for fecal pH of animals on the modified diets, are not especially different from those obtained with the basal diet, in respect not only to irregularity but also to range of pH values.

It would appear, therefore, that the addition in amounts equivalent to 2 per cent sodium carbonate, of acid- or base-forming salts to the Steenbock and Black rachitogenic diet produces no effect not shown by the basal diet alone. The failure of alkali to induce or promote rachitogenesis in this manner is supported by Shelling

(17), but is contrary to the findings of McClendon (18), and of Zucker, Johnson, and Barnett (6). The observation of the latter workers that calcium chloride tended to counteract the development of rickets when it replaced a neutral calcium salt in a rachitogenic diet, is not in accord with the findings of McCollum, Simmonds, Shipley, and Park (19) who observed no difference in effect between the chloride and carbonate. Shohl, Bennett, and Weed (20) fed a modified Steenbock and Black diet containing added phosphate in different forms so that the

TABLE I.  
*pH along Intestinal Tract of Normal and Rachitic Albino Rats.*

Rat No.	Normal.				Rat No.	Rachitic.			
	Upper ileum.	Lower ileum.	Cecum.	Colon.		Upper ileum.	Lower ileum.	Cecum.	Colon.
2002	6.8	7.1	7.3	6.85	2003	6.5	7.3	7.4	7.55
2008	6.5	7.2	7.8	7.3	2006	6.3	7.7	7.5	7.6
2064	6.2	6.9	7.35	7.05	2069	6.4	6.75	6.7	7.5
2065	6.3	6.6	6.7	6.5	2080	6.2	7.3	7.45	7.55
2066	6.3	6.6	7.05	6.55	2081	6.4	6.5	7.2	7.15
2067	6.3	6.8	7.2	6.5	2082	6.2	6.85	7.5	7.4
2072	6.2	7.3	7.4	7.25	2083	6.2	7.5	7.5	7.55
2073	6.5	6.5	7.15	6.5	2092	6.4	7.0	7.45	7.65
2075	6.3	6.8	7.05	6.5					
2077	6.4	6.85	7.05	6.85					
2086	6.2	7.0	7.25	7.0					
2088	6.2	6.4	7.2	6.6					
2090	6.6	7.3	7.45	7.2					
Average.	6.37	6.87	7.22	6.82		6.32	7.11	7.34	7.42

potential reaction was acid, neutral, or alkaline, and showed that the acid diet was the least conducive to bone deposition.

#### *Reaction along the Intestinal Tract.*

Since no particular difference was observed among the groups on the various salt modifications of the basal diet, the pH data have been grouped together in Table I. The division has been made according to whether the rats were found to be rachitic at autopsy, or cured of this condition. Normal controls are also included in the latter division.

The data indicate that on the average there is practically no difference between normal and rachitic rats in the reaction of the contents of the upper ileum. The alkalinity tends to increase along the intestinal tract, the average alkalinity being somewhat higher in rachitic rats, until a maximum is reached in the cecum.

In the normal rat the pH of the contents of the colon falls slightly but definitely below the value in the cecum, whereas in the rachitic animal this tendency is not observed. With few exceptions the reaction appears to suffer little change from that of the cecal contents. Similar findings may be seen in the data of Redman, Willimott, and Wokes (10) and of Yoder (11). The averages reported by Wokes and Willimott (21) show an increase in the pH beyond the cecum in rachitic rats, but not in normal animals. The observations of Grayzel and Miller (8) show a progressive increase in pH in both normal and rachitic dogs from the duodenum to the colon.

These data appear to lend support to the view that the small intestines exhibit relatively little functional change in rickets, and since this is the site of calcium absorption, the latter is probably not of prime etiological importance. Bergeim (22) has demonstrated reabsorption of Ca and P in the lower bowel of the normal animal, resulting in positive balances. This has been shown to be associated with lowered alkalinity. In the rachitic animal, however, excessive excretion of Ca and simultaneously of P, continues to take place in the lower bowel. Here the alkalinity continues to remain high. A condition of acidity where absorption preponderates over excretion, and of alkalinity where the reverse is true, has been regarded as a causal relation by Bergeim also (personal communication).

Evidence obtained along other lines would seem to add support to the importance of excessive P elimination as an etiological factor in rickets. It has been observed (23) that the increased retention of Ca and P produced by ultra-violet radiation in children, does not necessarily imply increased absorption, but may indicate that in rickets excessive excretion occurs. In studies of the metabolism of rachitic dogs, Shohl and Bennett (24) found lower balances of P than of Ca. Greater retention of Ca than of P was also observed in rats by Karelitz and Shohl (25). Calcification has actually been shown to take place in rickets (26),



but at a rate too slow to keep pace with the growing osteoid tissue. Unless it is complicated with tetany, in rickets there is usually a normal serum Ca, but the values for inorganic P are low (27). Subcutaneously injected calcium has been demonstrated not to be retained by the rachitic animal (23).

When facts such as these are coordinated, it appears that rickets is not due to defective Ca absorption primarily, but rather to a failure properly to reabsorb P. Inasmuch as excretion of P into the small intestine appears to favor the absorption of Ca (22), it is further possible that the recalcification observed in fasted rachitic animals may be due to the diminished need for excretion of P to facilitate Ca absorption, and hence the restoration of a higher level of inorganic P in the blood.

#### CONCLUSIONS.

Aside from its lack of specificity, it would seem that the fecal pH test for vitamin D potency fails to approach a quantitative ideal for these reasons: There is not sufficient uniformity in the reaction of the feces to permit the establishment of a border zone to distinguish between normal and rachitic rats; there is considerable fluctuation from day to day among individual rats in the fecal pH value, even while the diet remains unchanged; the time response of different rats to the experimental diets is not sufficiently constant.

As a qualitative indication of the presence of vitamin D the test is satisfactory provided other factors which influence the reaction (such as Ca:P ratio, acid:base ratio, inanition, *etc.*) are controlled.

The addition of acidogenic or alkaligenic salts to the Steenbock and Black rickets-producing diet, in the amounts used by Zucker, Johnson, and Barnett (6), was without effect on either the promotion or inhibition of rickets in these experiments. Although these salt diets appeared to possess no influence on the intestinal pH values not shown by the unmodified basal diet, a positive effect of greater proportions of the salts must not be excluded.

The decreased alkalinity of the colonic contents of the normal rat is regarded as related to phosphorus reabsorption which normally occurs at that portion of the intestinal canal; whereas the sustained increase in alkalinity at this region in the rachitic

rat appears to be associated with inadequate phosphorus reabsorption. Since even the rachitic rat has been shown to absorb and utilize calcium to a certain degree, it is probable that the primary etiological disturbance in rickets is the loss of phosphorus from the lower bowel, and the consequent decrease in the level of this element in the blood plasma. Fasting, being accompanied by inadequate calcium intake, may affect recalcification because of the conservation or restoration of the inorganic phosphorus level in the "bathing fluid" of the bones.

*Addendum.*—Following the preparation of this manuscript, a paper by Shohl and Bing (28) appeared, in which similar conclusions are reached regarding the value of the fecal pH test in rickets. It is interesting to note their failure to reduce fecal pH by irradiation of a modified Steenbock diet.

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## THE EFFECT OF SCURVY-PRODUCING DIETS AND TYRAMINE ON THE BLOOD OF GUINEA PIGS.

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In 1914<sup>1</sup> Iwao published observations on three guinea pigs, from which he concluded that the subcutaneous administration of tyramine leads to a profound anemia and a blood picture resembling that observed in pernicious anemia. These experiments were repeated on a much larger scale in 1920 by Koessler and Harris<sup>2</sup> with entirely negative results. An anemia was not obtained in any of the animals.

A careful consideration of the article by Iwao indicates that his animals may have been nourished on a deficient diet. Their diet consisted of okara, which is the residue left after finely ground soy beans have been extracted with cold water to remove the legumin and other soluble proteins. The precipitated legumin (tofu) is used as an article of diet in Japan. The okara is used for feeding animals.

Since whole soy beans are certainly deficient in vitamins C and A,<sup>3</sup> it is inconceivable that okara can, by itself, serve as a maintenance diet for guinea pigs. We thought, therefore, that the results obtained by Iwao might be the composite effect of the toxic action of tyramine and a diet deficient in vitamins; or the anemia and the blood picture might, of course, have been the

<sup>1</sup> Iwao, T., *Biochem. Z.*, 1914, lix, 436.

<sup>2</sup> Koessler, K. K., and Harris, R. L., unpublished data cited in Wells, H. G., *Chemical pathology*, Philadelphia, 5th edition, 1925, 342.

<sup>3</sup> Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 369. Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, 1919, xxxvii, 201. Givens, M. H., and Cohen, B., *J. Biol. Chem.*, 1918, xxxvi, 127. Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

direct result of a deficient diet. These possibilities have been subjected to experimental investigation.

#### EXPERIMENTAL.

*Group 1.*—Five guinea pigs with an initial weight of 350 to 420 gm. were fed exclusively on okara, which is the diet that Iwao claims to have fed to his animals. The animals died after 27, 29, 28, 24, and 29 days respectively. They all showed a typical scurvy picture and none of them developed an anemia.<sup>4</sup> Abnormal erythrocytes developed after the okara had been fed for 20 days. These increased in number rapidly so that, just before death, 15 per cent of the cells were polychromatophilic, 20 to 25 per cent were reticulated, and 0.2 per cent were nucleated. Poikilocytosis and anisocytosis were marked.

It is obvious from these results that okara could not have been the *only* foodstuff employed by Iwao as a maintenance diet. Of particular note is the abnormal red blood picture which resembles that described by Iwao, excepting for the absence of an anemia. This blood picture was attributed, by Iwao, to the tyramine.

*Group 2.*—Four guinea pigs with an initial weight of 365 to 430 gm. were placed on a diet that consisted of 96 per cent autoclaved whole soy beans, 1 per cent ferric citrate, 1 per cent NaCl, and 2 per cent Ca lactate. The animals lived 28, 26, 33, and 32 days respectively. They all showed a typical scurvy picture and one of them developed an anemia.

The blood findings in this group of animals were almost identical with those just described for okara. In addition to the production of abnormal erythrocytes, one of these animals developed an anemia. This animal lived 28 days. The erythrocyte count remained normal (5.9 million) for 11 days, then declined rapidly reaching a low value of 2.9 million just before death. The hemoglobin content was reduced from 15.2 to 8.5 gm. The blood picture showed abnormalities that were qualitatively and quantitatively identical with those described under Group 1.

A soy bean diet regularly produces an abnormal red blood picture just before the animals die with scurvy symptoms. Occas-

<sup>4</sup> Blood was drawn twice a week from all of our experimental animals. The examination included a complete differential white and red blood cell count and a hemoglobin determination.

ionally, it seems, an anemia is also produced. The anemia appears to be the exception rather than the rule.

*Group 3.*—Six guinea pigs with an initial weight of 356 to 487 gm. were placed on a diet consisting of 83 per cent autoclaved soy beans, 1 per cent ferric citrate, 1 per cent NaCl, 2 per cent Ca lactate, 3 per cent lemon juice, and 10 per cent coconut oil. This diet should contain all of the vitamins, excepting vitamin A, in abundance. The animals divided themselves rather sharply into two groups. Four of them died of pneumonia in from 49 to 60 days; the other two lived on for 75 days, the time of concluding the experiment. The first four gained in weight for some time and then lost rapidly, became infected, and died. The other two animals gained in weight rather unsteadily during the entire 75 day period. None of these animals became anemic and none of them showed an abnormal red blood picture.

*Group 3 A.*—This group of four animals completes Group 3. The diet was identical with that just described. In addition these animals received daily subcutaneous injections of tyramine hydrochloride exactly as described by Iwao, the dose being 2 mg. of tyramine per 100 gm. of body weight. None of the animals became anemic. The red blood picture remained normal. One of the animals died of bronchopneumonia; the other three lived on until the experiment was discontinued.

*Group 4.*—This group of five animals received a diet identical with that described under Group 3 excepting that 3 cc. of orange juice replaced the 3 cc. of lemon juice. This diet should contain an ample amount of all of the vitamins. Three of these guinea pigs gained in weight slowly and steadily over a period of 75 days. Two of them died with a mastoid infection after 30 days. The red blood picture was entirely normal in all of these animals. Tyramine injections were not carried out on this group.

*Group 5.*—These seven animals were fed oats and carrots. They gained in weight rapidly and showed an entirely normal blood picture throughout.

*Group 5 A.*—This group of seven animals was fed oats and carrots. Subcutaneous injections of tyramine—2 mg. of tyramine per 100 gm. of body weight—were given daily. The red blood picture remained normal in all of these animals. The animals continued to gain in weight steadily.

*Group 6.*—One animal was subjected to a series of diet changes with soy beans and minerals as constant dietary constituents. A marked scurvy was produced at one time which was cured by administration of lemon juice. The scurvy period was associated with blood changes identical with those already described. There was no anemia. The abnormalities disappeared soon after the lemon juice feeding was inaugurated. This animal lived for 4.5 months. The first evidences of vitamin A deficiency were noticeable after 3 months. A rapid decline in weight occurred during the last 15 days. Tyramine was injected daily during the last 30 days.

This animal was not anemic at any time. There were no abnormalities in the red blood picture excepting for the brief period during which marked scurvy symptoms were evident.

#### CONCLUSIONS.

All of the results obtained are unidirectional. Tyramine injected subcutaneously into guinea pigs does *not* produce nor lead to anemia either in well fed guinea pigs or in animals that are fed deficient diets. There is no evidence, in these experiments, that, in guinea pigs, a diet deficient in vitamin A is conducive to anemia nor to the production of abnormal erythrocytes. A diet consisting exclusively of autoclaved soy beans and minerals rapidly leads to scurvy symptoms and death. In such animals an abnormal red blood picture is invariably obtained. There is present a marked polychromatophilia, anisocytosis, and poikilocytosis. The smear may contain a high percentage of nucleated red cells. Reticulocytes may be present in quantities up to 25 per cent. The abnormal red blood picture may, occasionally, be associated with an anemia.



# STARCH HYDROLYSIS AS AFFECTED BY POLARIZED LIGHT.

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## I.

The papers published by Baly and Semmens (1, 2) and Semmens (3, 4) were the first of a number dealing with the action of polarized light on biological processes. They were notable for the fact that they described very striking effects of plane polarized light in accelerating hydrolysis of starch, even when the experiment was performed in the crudest way.<sup>1</sup> Among the various papers published more recently, two very divergent lines of results are found: on the one hand, Macht and his coworker (7), Bryant (8), Morrison (9), and Bhatmagar and Lal (10) confirm the results of Baly and Semmens; while Jones (11), and Bunker and Anderson (12), on the contrary, were unable to duplicate Baly's results. It should also be noted that, starting from a somewhat different standpoint, Crozier and Mangelsdorf (13), in an attempt to find a physiological effect of polarized light in tropistic reactions, obtained no significant differences between responses to ordinary and to polarized lights.

In most of the papers concerned with starch hydrolysis, details concerning the set-up and technique are given in a very simple way; in many cases, too, it can be shown from what has been published that certain sources of error were not avoided; furthermore, in most of the cases, polarized light was obtained by the use of piles of glass plates and frequently not enough attention has been paid to the properties of the light after passage through such piles of glass; finally, no indications have been given as to the type of enzyme used and of the real activity of the enzyme (in certain cases, even, it is merely spoken of as "very dilute solution" and "concentrated solution" of diastase). These facts induced us

<sup>1</sup> The discussions of Knauthe (5) and by Kofoed (6) are on a purely hypothetical basis and cannot be considered as dealing with the intrinsic effect of polarized light; too many factors were uncontrolled.

several years ago to attempt an elucidation of certain points related to this question, since the results might be significant for the problem of sensory stimulation by light (cf. Crozier and Mangelsdorf (13)).

## II.

The method used was to radiate given volumes of a mixture of starch and amylase solutions by ordinary and by plane polarized light, of equal intensities, and to keep an aliquot portion of the stock mixture in the dark as a control, all other conditions being identical. The different factors which were thus under control were: light and conditions of radiation, temperature during radiation, and composition of the radiated mixture.

*Light and Conditions of Radiation.*—The light used in these experiments was obtained from 100, 150, and 500 watt unfrosted Mazda bulbs. The light was passed through a cell, with parallel walls, containing a dilute aqueous solution of cobalt nitrate to remove the infra-red and the ultra-violet parts of the spectrum. We were using, in this way, only the visible part of the spectrum, ranging from  $430\text{ }\mu$  to  $680\text{ }\mu$ , and the conditions so obtained were duplicated without any trouble.

The beams of light, after filtration through the cobalt nitrate solution, were passed, in the case of the polarized beam, through a large nicol prism and allowed then to be totally reflected by the silvered surface of a plate glass placed at  $45^\circ$ ; the beam of ordinary light was directly reflected by an identically silvered surface. Care was taken not to use ordinary mirrors, where the beam of light would have to pass through the thickness of the plate twice. Silvered plates were made, with the highly polished silver surface directly reflecting the light. Both mirrors were tested in the experimental position for additional polarization, but none could be detected. The position of the nicol prism was also changed by rotating it  $90^\circ$  around its axis. No difference was noticed in the effect on hydrolysis.

The two kinds of light after total reflection impinged on the bottoms of conical flasks of Pyrex glass selected with very flat bottoms; due control had shown that the flasks used did not introduce any additional polarization effects.

All extraneous light was excluded by an enclosing dark chamber,

and the positions of the lamps were chosen so as to obtain perfect equality of illumination for the two samples; the equality point was determined with a Macbeth illuminometer. The light emitted by the lamps was also analyzed with a Glan spectrophotometer and the curves relating the intensities of illumination in the different parts of the spectra will be found in Fig. 1.

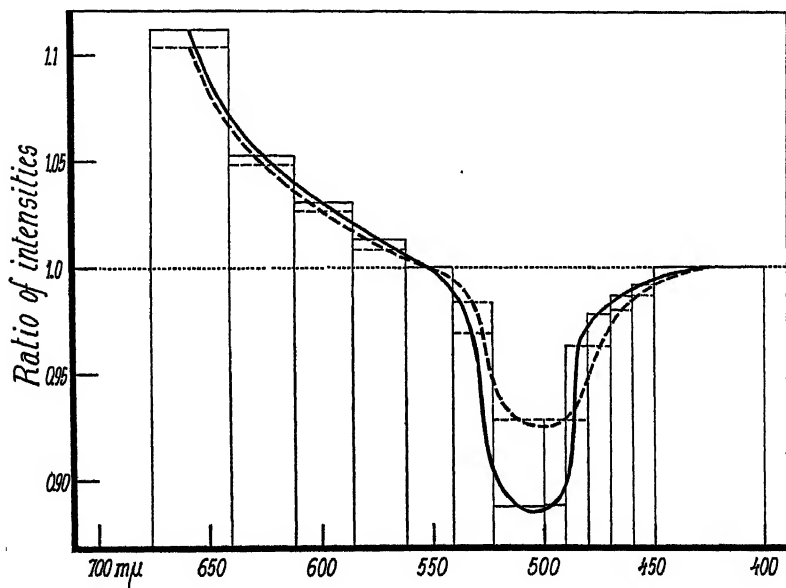


Fig. 1. Intensity distribution in the spectra of the lights used. Plain line = 267 meter-candle lamp; broken line = 50.6 meter-candle lamp. The comparison was made with a Glan spectrophotometer, the polarized beam being used as the standard (taken as 1.0) at each wave-length; the ordinate scale therefore gives relative luminous intensities of the two sources used, as a function of wave-length.

In these experiments two intensities of illumination were used: 50.6 meter-candles and 267 meter-candles. The illuminations refer to the light actually coming into the systems studied. In a number of experiments, the light filters were mounted as windows of the thermostat. Checks showed that this modification did not introduce any special effects.

*Temperature during Radiation.*—All the hydrolyses were per-

formed at  $22.5^{\circ}$ , this temperature being selected after preliminary tests as the most convenient on account of the relative rates of hydrolysis. We used an air thermostat (Fig. 2) in which during a single experiment no variation in temperature of more than  $\pm 0.25^{\circ}$  was found. A space was provided in the same thermostat for the dark control; no radiation of this sample was possible. Furthermore, the whole thermostat was kept in a room in which variations in temperature never exceeded  $1.5^{\circ}$  as a maximum. The scheme used has the advantage of having all the hydrolyses occurring at the same temperature for the different samples of any one run.

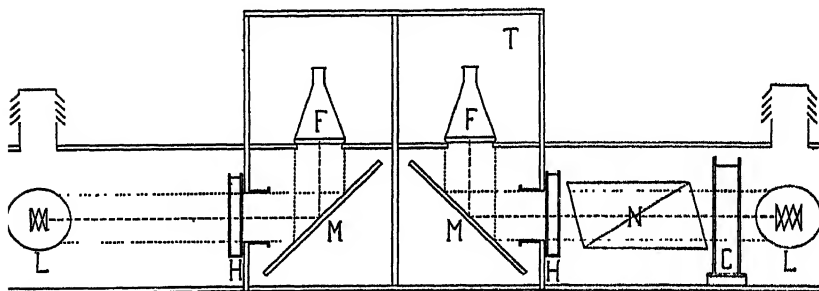


FIG. 2. Illuminated air thermostat and accessories. *T*, thermostat; *F*, flasks where radiated hydrolyses are performed; *H*, cobalt nitrate heat filter; *C*, water cell as additional heat filter; *M*, mirrors; *N*, nicol prism; *L*, lamps.

*Composition of Radiated Mixture.*—The substratum subjected to hydrolysis was a 1 per cent solution of Lintner soluble starch made up in distilled water by boiling for 3 minutes and completing to initial volume with boiling distilled water.

The enzyme used was prepared from "Merck's diastase of malt—medicinal U.S.P. IX. 22467;" 1 gm. of the enzyme preparation was dissolved in 100 cc. of a 1 per cent NaF solution. This solution, kept for 2 hours at  $0^{\circ}$  with occasional stirring, is then centrifuged for 5 minutes at 1800 r.p.m., cooled at  $0^{\circ}$  again, and freed from starch and other substances by precipitation with alcohol, the volume of alcohol added making the alcoholic titer of the mixture 50 per cent. This first precipitate is separated and discarded. The enzyme is then precipitated by making the alco-

holic titer of the solution 65 per cent; it is then dried carefully in a desiccator. All these manipulations of the enzyme, as well as the subsequent solution of it, are carried out in the dark in order to avoid any possible effect of light (destruction or inhibition). As will be shown later, very definite effects even of dim light are noticeable. 1 gm. of the crude preparation yields about 0.470 gm. of a white powder, very easily and completely soluble in water, freed from starch, dextrins, *etc.*, as well as from maltase. The solution of this very active diastase has to be made up as needed and keeps only for a very short time at ordinary temperature; up to 5° on the contrary it is relatively stable in the dark, especially if buffered with the McIlvaine phosphate-citrate mixture to a pH of 5.0. It should be noted also that the addition of starch solution to the enzymatic solution "protects" it to a considerable extent against this spontaneous destruction.

All our hydrolyses were made at pH 5.0, as a few preliminary trials had shown the enzyme to exhibit a maximum activity at that point. 60 cc. of soluble starch solution (1 per cent) were buffered with 10 cc. of the adequate McIlvaine mixture, and 5 cc. of a 1 per cent enzyme solution buffered to the same point was added. Both the starch and the buffer solutions were always kept in a thermostat at 22.5° before the addition of enzyme, so as to be able to reckon the time of mixing as the starting point for the hydrolysis.

The 75 cc. volume was divided immediately into three equal portions, two of which were radiated, the third being kept in the dark. The duration of radiation was varied from 1 minute to 9 hours, and in each case (for each length of exposure) at least five similar runs were made. The titration of the maltose formed was done on the total mass of mixture exposed to the action of light. This procedure seemed to us superior to that where aliquot portions are removed after definite time periods; no change in quantity of substrate or of the ratio of enzyme to substrate could then introduce any interference.

Since preliminary tests had shown that no glucose was present at any time, even for very prolonged hydrolyses, we may consider the reducing substances as maltose, especially in view of the fact that the relative reducing power of dextrins and hexosans present

is always small. At the end of the radiation period, the enzyme was destroyed by instantaneous immersion of the three flasks in boiling water, where they were kept for 3 minutes. The time error so introduced in the total length of exposure is, at a maximum, 30 seconds. The quantitative determination of the hydrolytic products was carried on by polarimetric and reductimetric methods. Polarimetric readings were made with the Laurent-Pellin polarimeter (large model) after addition of 0.1 in volume of a 5 per cent sodium carbonate solution to stop mutarotation, with the green line of the mercury arc as the source of monochromatic light.

The reductimetric measurements were made with Benedict's quantitative solution ((14), p. 883), all the reducing substances present being calculated in terms of maltose. In most of the later determinations Bertrand's modification was used ((15), p. 98). The two methods used gave results which were perfectly consistent one with the other. We discarded the iodometric titration of dextrins, after a thorough test, as being too unreliable for quantitative measurements. The fading effects and the time factor play rôles which are not well enough known to permit adequate corrections.

As we found in later tests, even the addition of 25 cc. of a solution containing 0.03 gm. of iodine and 0.06 gm. of KI per liter (as used by Bunker and Anderson (12, p. 483)) does not stop hydrolysis immediately.

### III.

The only further modification made in the technique described was to radiate separately the enzyme solution, buffered or not, and the starch solution, buffered or not, before allowing hydrolysis to go on in the dark or light.

At 22.5° and with 267 meter-candles the radiation of the enzyme solution rapidly reduces the activity of the enzyme to very small values. At 0°, on the contrary, the hydrolytic power is not destroyed and the phenomena which will be described in detail later on are shown for a threshold radiation of 9 minutes. For 15 minutes of exposure at 0° the maximum effect obtainable is found and longer durations of radiation do not accentuate this effect.

## IV.

*Hydrolysis in Ordinary and in Polarized Light.*

The first sets of experiments were parallel runs, with radiation by the two types of light at intensity 50.6 meter-candles, a third flask being kept in the dark as a control. These runs departed from the later ones by the fact that the enzyme was *not* prepared

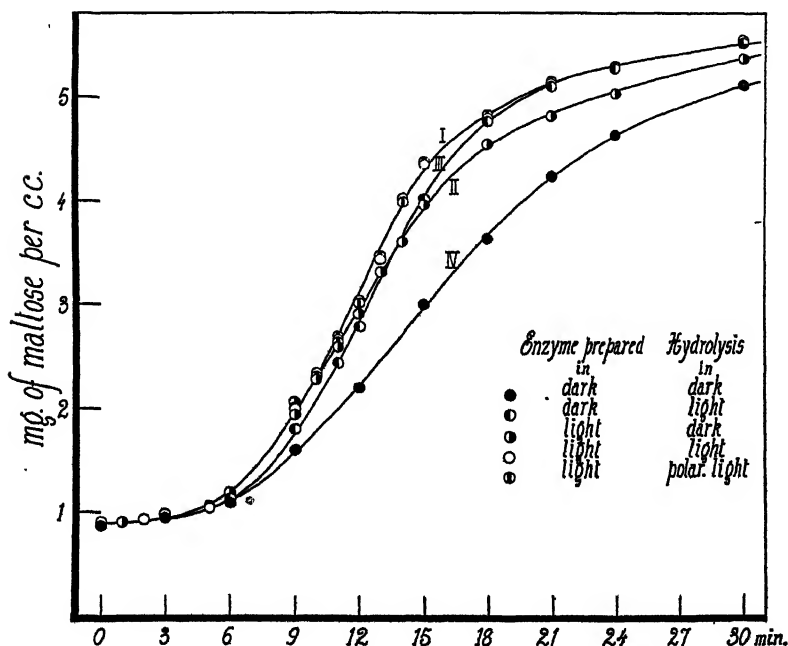


FIG. 3. The course of the hydrolysis of soluble starch by purified diastase under various conditions of illumination, during the first 30 minutes.

in the dark; all the other conditions of preparation were nevertheless as described previously.

These experiments showed that the ordinary and the polarized lights had a "stimulating" effect on the hydrolytic process, and both of about the same magnitude. When hydrolysis goes on (Curves I and II in Figs. 3 and 4) in the presence of light, a larger amount of the starch substrate is hydrolyzed. The differences

between the two curves (ordinary light and polarized light) are quite insignificant; they are of the magnitude of twice the probable error of the mean and so far as it is consistent it can be attributed to the slight differences existing in the spectral composition of the lights.

To magnify the differences which might exist, the intensity of illumination was brought to 267 meter-candles. Sets of experi-

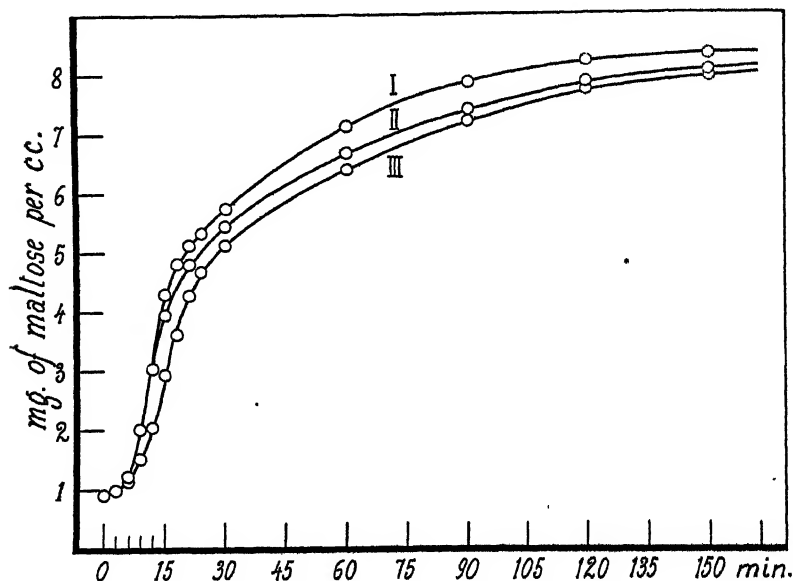


FIG. 4. The course of the hydrolysis of soluble starch by purified diastase under various conditions of illumination, as in Fig. 3 but over a longer time interval. Curve I, hydrolysis in light (polarized or not); Curve II, hydrolysis in dark; Curve III, enzyme prepared in the dark, hydrolysis in the dark.

ments done under these conditions (the enzyme being always prepared under ordinary laboratory illumination) showed the same type of relative difference, the absolute values alone being increased.

Thus, differences are found between hydrolysis going on in dark and in light; in light the hydrolysis goes farther than in the dark for equal durations of reaction. As to a difference between effects



of ordinary light and of polarized light, none could be detected which can be considered significant.

This point is of especial interest in view of the facts described by several authors, Pincussen (16), Jodlbauer and von Tappeiner (17), and others, who have found an *inhibitory* effect of light on diastase. But it can be pointed out that these authors have not given enough indications concerning the preliminary treatments of the enzyme. In the conditions here described the stimulating effect was obtained without exception and with a remarkable consistency.

In all the experiments hitherto reported by different authors, no attention has ever been paid to the fact that the enzyme was prepared in an illuminated room (the intensity of this illumination never being recorded). Do differences occur if the entire purification of the enzyme is carried out in dark?

We had noticed that aliquot parts of an enzyme solution which had been kept between two successive runs in an ice box, on the one hand, and in melting ice in the laboratory on the other hand, showed a very peculiar difference in behavior. The sample kept in the laboratory had a higher hydrolytic power for all durations of reaction. Systematic preparation in the dark was then performed, as described under "Technique". The utmost care was taken to avoid all light during purification. Measurements and mixing of the enzyme with the starch solution were done under illumination with a 40 watt bulb and a Wratten Safelight filter series 2, avoiding as much as possible all direct illumination.

If we now repeat the radiated hydrolysis under these new conditions, we find that the curve describing the amount of maltose formed at each moment is different from the one from the first sets of experiments. All the points of this new light curve (Fig. 3, Curve III) lie under the light curve of the first group, up to a point corresponding to 18 to 19 minutes of radiation, where the two curves fuse and are thereafter identical.

On the other hand the sigmoid curve corresponding to the dark phenomenon (Fig. 3, Curve IV) is much flatter and stays normally below the light curves just as in the first group. All these curves tend to reach the same equilibrium level; apart from their special character at the start, their differences are in the times required to reach the equilibrium level.

One more point should be added. Comparing the two groups

of experiments, Group 1, light and dark reaction with enzyme prepared in light, Group 2, light and dark reaction with enzyme prepared in dark, we may notice that within each group the two curves run together up to a certain point; 10 minutes for Group 1, 6 minutes for Group 2. In each group there is a threshold value for the radiation effect, the threshold being lower for Group 2. Below this threshold time of exposure to light there is no specific difference between "dark" and "light" reactions.

In the system starch-diaxase what is the part responsible for such phenomena?

Tests were conducted where the two constituents were radiated separately: the enzyme at  $0^{\circ}$  in distilled water or in buffered solution, the starch at  $22.5^{\circ}$  in buffered solution or without buffer. We have seen that at  $22.5^{\circ}$  the enzyme suffers photodestruction. The hydrolysis was carried on in the dark after mixture of the two constituents.

The radiation effect is shown only when the enzyme solution is radiated. If we now vary the length of time during which the enzyme is illuminated, we again find a threshold value. Up to 9 minutes no differential result is found in comparing illuminated enzyme with that not illuminated. It requires 9 minutes of exposure to get the effect, and by increasing the time little by little we increase in a larger proportion the difference until the total effect, which can be calculated from the curves, is obtained with a 15 minute exposure. Further increase of the exposure period does not increase the discrepancy between the two curves, which remain practically constant for a very long time. One of the main points in these curves is the presence of an apparent lag period at their start. This could be accounted for by different explanations. But before any theoretical discussion can be carried out, further analyses have to be made again.<sup>2</sup>

<sup>2</sup> All these curves form a family of sigmoid curves, reminding us very much of growth curves described for numerous organisms. This fact seemed to depart also from the usually accepted form of hydrolytic curve, especially by the presence of a lag period at the start. Attempts made to fit these curves by a first order process equation fail completely, as do attempts made after introduction of a factor involving autocatalysis, even with correction for initial reducing power. It seems that the process cannot be of first order type, even autocatalyzed. A second order autocatalytic process, on the contrary, seems to describe better the course of events. Other tests will be made for the case here described.

The writers desire to acknowledge their appreciation of assistance received from Professor T. Lyman, of the Jefferson Physical Laboratory and from Professor E. S. Larsen, of the Department of Mineralogy, during the course of these experiments.

#### SUMMARY.

The situation regarding the effect of radiation on the starch-diastase system is, in brief:

1. Ordinary light and polarized light, of the same intensity and as closely as possible similar in spectral composition, have the same effect.

2. Light falling on the starch-diastase system as described, increases the rate of hydrolysis over that of the same reaction in the dark.

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## QUANTITATIVE STUDIES OF $\beta$ -OXIDATION.

### II. THE METABOLISM OF PHENYLVALERIC ACID, PHENYL- $\alpha$ , $\beta$ -PENTENIC ACID, PHENYL- $\beta$ , $\gamma$ -PENTENIC ACID, MANDELIC ACID, PHENYL- $\beta$ -HYDROXYPROPIONIC ACID, AND ACETOPHENONE IN DOGS.\*

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In a previous paper (1) it was shown that benzoic acid whether fed or derived from the oxidation of cinnamic acid or phenylpropionic acid is conjugated in the dog with glycuronic acid and glycine in the ratio of 3:1, as contrasted with the ratio of 1:2 with which phenylacetic acid is combined, irrespective of whether it is fed or produced from phenylbutyric acid or phenylisocrotonic acid. Because of the possible importance that these studies may have in elucidating certain phases of the metabolism of fatty acids, it was deemed desirable to extend this investigation. In the former publication the metabolism of the acids of the phenyl-substituted fatty acid series up to and including those having 4 carbon atoms in the aliphatic chain were investigated. In this paper the work is extended to members of the series having a 5 carbon chain. Besides these the oxidation and conjugation of three other compounds, namely mandelic acid, phenyl- $\beta$ -hydroxypropionic acid, and acetophenone, were quantitatively studied in the hope of getting more data which might be useful in throwing light on the question of the intermediary compounds formed in the catabolism of the fatty acids.

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

## EXPERIMENTAL.

The procedures employed in the experiments recorded in this paper were the same as those described in the previous publication (1). The analytical methods likewise were essentially the same except for the determination of phenylmethylcarbinol glycuronic acid. This substance was determined polariscopically with the constant  $[\alpha]_D^{20} = -124^\circ$  of the potassium salt of this acid (2). Attempts to determine this compound, like menthol glycuronic acid, failed because the acid is relatively insoluble in ether and therefore cannot easily be quantitatively extracted with that solvent. For the determination of phenyl- $\beta$ -hydroxypropionic acid excreted unchanged, the urine was extracted with ether which removed both the hydroxy acid and hippuric acid. After evaporation of the ether, the residue was extracted with chloroform which dissolved the hydroxy acid but not the hippuric acid. The weight of the residue remaining after the chloroform was evaporated represented the amount of phenyl- $\beta$ -hydroxypropionic acid appearing in the urine.

*Preparation of Materials.*

*Phenylvaleric Acid.*—This acid was prepared by reducing cinnamylidenemalonic acid directly with constant boiling hydriodic acid. Dakin's method (3) for preparing cinnamylidenemalonic acid is very satisfactory, for it is simple and the yield is high. The procedure consists in condensing cinnamic aldehyde with malonic acid in the presence of a trace of aniline which serves as a catalyst. The old way, however, of reducing cinnamylidenemalonic acid to phenylvaleric acid is not only long and tedious but the yield is very unsatisfactory. The compound is first reduced to phenylpropenylmalonic acid by means of sodium amalgam. This latter compound on boiling with water loses carbon dioxide and is changed to phenyl- $\beta$ ,  $\gamma$ -pentenic acid. Since this compound is not reduced by sodium amalgam, it must be boiled with sodium hydroxide solution for 24 hours, since under those conditions about half of the compound is changed to the phenyl- $\alpha$ ,  $\beta$ -pentenic acid which can be reduced to phenylvaleric acid. Since it was found that cinnamylidenemalonic acid can be directly reduced to phenylvaleric acid by means of concentrated hydriodic

acid and red phosphorus, all the irksome steps of the old procedure were eliminated, and the preparation of phenylvaleric acid made exceedingly simple. The actual procedure was as follows: 1 part of cinnamylidenemalonic acid was mixed with 1 part of red phosphorus and 10 parts of concentrated hydriodic acid. The reduction was carried out in a Kjeldahl flask into the neck of which was inserted a large test-tube through which water was circulating. After the mixture was refluxed for 8 to 10 hours the intense yellow color of the solution changed to a straw-color indicating that the reduction was complete. After cooling the solution, the hydriodic acid was decanted from the gummy mass consisting of the desired product and red phosphorus. To remove the remaining hydriodic acid, the residue was extracted several times with hot water, and then treated with ether which dissolved the phenylvaleric acid. The ether solution was next treated with dilute sodium thiosulfate solution to remove traces of free iodine. On evaporating off the ether, a yellow oil remained which on standing slowly changed to a crystalline mass. After distilling the oil under reduced pressure, a crystalline product was obtained which was sufficiently pure for feeding experiments. By recrystallizing from hot water, a colorless product having the correct melting point ( $59^{\circ}$ ) was obtained.

*Phenyl- $\beta$ ,  $\gamma$ -Pentenic Acid.*—This compound was prepared by the method used by Dakin. It consists in the reduction of cinnamylidenemalonic acid by means of sodium amalgam, isolating the reduced acid, phenylpropenylmalonic acid, which on boiling with water loses carbon dioxide and is completely converted to phenyl- $\beta$ ,  $\gamma$ -pentenic acid. It was not possible, however, to follow exactly the directions given by Thiele and Meisenheimer (4) for these authors claim complete reduction of 100 gm. of cinnamylidenemalonic acid with 160 gm. of 3 per cent sodium amalgam, whereas in this study it was found that 35 gm. of the acid required 270 gm. of 2 per cent sodium amalgam. Phenyl- $\beta$ ,  $\gamma$ -pentenic acid was obtained in the form of a light yellow oil which did not crystallize on standing. It was used in the recorded experiments without further purification.

*Phenyl- $\alpha$ ,  $\beta$ -Pentenic Acid.*—This compound was obtained by the method of Fittig and Hoffmann (5). By boiling phenyl- $\beta$ ,  $\gamma$ -pentenic acid with 10 per cent sodium hydroxide solution for

24 hours about 50 per cent of the acid is converted to phenyl- $\alpha$ ,  $\beta$ -pentenic acid which can easily be isolated and obtained in pure crystalline form. The product obtained by this method corresponded in physical properties to the substance described by Fittig and Hoffmann.

*Phenyl- $\beta$ -Hydroxypropionic Acid.*—The acid was prepared according to the directions of Fittig and Binder (6). Cinnamic acid was treated with hydrobromic acid saturated at 0°. The resulting phenyl- $\beta$ -bromopropionic acid was boiled with 10 volumes of water whereupon the bromo compound was converted to the corresponding hydroxy acid.

#### DISCUSSION.

The results obtained on the catabolism of phenylvaleric acid as well as of the two phenylpentenic acids are in harmony with the observations made on the lower members of the phenyl aliphatic acid series previously reported. In accordance with the hypothesis of  $\beta$ -oxidation, phenylvaleric acid as well as the two unsaturated acids having 5 carbon atoms, yields only benzoic acid. Knoop (7), who was the pioneer in the systematic investigation of the metabolism of the phenyl-substituted aliphatic acids, stated that only hippuric acid was found in the urine after feeding phenylvaleric acid to dogs. Dakin (3) corroborated these findings and extended his study to the various pentenic acids and found that these likewise, irrespective of the position of the double bond, yielded benzoic acid (hippuric acid) but not a trace of phenylacetic acid. As stated in a previous paper, both Knoop's and Dakin's work was primarily qualitative, and neither investigator mentioned glycuronic acid. That phenylvaleric acid is oxidized exclusively and quantitatively to benzoic acid has been pretty definitely established by the recent work of Raper and Wayne (8). Their interest, however, was centered solely on the quantitative recovery of benzoic acid without delving into the question how the benzoic acid was conjugated. As brought out in Paper I of this series, the ratio with which benzoic acid is combined with glycuronic acid and glycine is remarkably constant, namely 3:1, and this ratio holds whether the benzoic acid is derived from phenylpropionic acid, cinnamic acid, or is fed directly. It seemed therefore of theoretical importance to determine whether the



benzoic acid derived from the metabolism of phenylvaleric acid would be conjugated in the same ratio as when it is produced from the shorter chain acids. If it is similarly conjugated, then the hypothesis of  $\beta$ -oxidation is further substantiated. From the results recorded in Table I, it can be seen that a similar but somewhat smaller ratio was obtained. Instead of the ratio of 3:1, a ratio of 2:1 was found. In other words, a somewhat higher percentage of the benzoic acid is combined with glycine. Nevertheless 66 per cent of the benzoic acid appears combined with glycuronic acid. The metabolism of phenylvaleric acid is distinctly different from that of phenylbutyric acid, since the latter is

TABLE I.

*Conjugation of Benzoic Acid Derived from Oxidation of Phenylvaleric Acid and of Two Phenylpentenic Acids.*

Dog 10; weight 8 kilos.

Substance fed.	Amount.	Equivalent in terms of benzoic acid.	Combined benzoic acid.			
			With glycuronic acid.		With glycine.	
			gm.	per cent	gm.	per cent
Phenylvaleric acid.....	6.0	4.10	2.03	66.5	1.03	33.5
“ “ .....	5.0	3.42	1.75	67.0	0.86	33.0
Phenyl- $\beta$ , $\gamma$ -pentic acid.....	7.0	4.85	2.38	67.5	1.15	32.5
“ “ .....	5.7	3.94	1.72	68.0	0.81	32.0
Phenyl- $\alpha$ , $\beta$ -pentic acid.....	5.0	3.46	1.57	64.5	0.87	35.5

oxidized quantitatively to phenylacetic acid which is conjugated with glycuronic acid to the extent only of 33 per cent. The metabolism of phenylvaleric acid, in spite of the small discrepancy in ratios, fits into the class of phenyl aliphatic acids having an odd number of carbon atoms. The surprising fact is not that there is some discrepancy in the ratios, but rather that such good agreement has been obtained. Phenylvaleric acid, it seems, is metabolized more slowly than the lower members of the series. This is indicated by the fact that the excretion of benzoic acid is less complete in the first 24 hours than it is in the case of the other acids previously studied. It has been repeatedly observed that when one has reason to suspect that the production of benzoic acid is slowed or delayed, the relative amount of hippuric acid is in-

creased. The slower metabolism of phenylvaleric acid would therefore account for the small increase of the conjugation with glycine. It seems safe to conjecture that as the molecular weights of the acids increase, the rate of catabolism will decrease, and therefore if the higher members of the phenyl aliphatic series were studied it is quite probable that the relative amount of hippuric acid would be further increased and the ratio of conjugation be progressively decreased. Raper and Wayne (8) found that in the case of the 9 and 10 carbon atom acids, the excretion of the end-products, benzoic acid and phenylacetic acid, was less complete. The authors are inclined to interpret these results as due to another metabolic mechanism besides  $\beta$ -oxidation. It is conceivable, however, that these results may be due either to a marked decrease in their metabolic rate, or even to a temporary storage. It is rather unfortunate that the conjugation of the end-products of these two higher acids was not recorded.

The metabolism of phenylvaleric acid as indicated by the conjugation of its end-product, benzoic acid, furnishes additional evidence for supporting the theory of  $\beta$ -oxidation. In the first place only benzoic acid, as required by the hypothesis, is produced. In the second place, the benzoic acid thus produced is conjugated with glycine and glycuronic acid in a ratio which approximates that obtained when benzoic acid is derived from the 3 carbon acids or is fed directly. Since phenylvaleric acid, because of its longer aliphatic chain, corresponds more closely to the normal fatty acids, the data obtained on its metabolism add considerable strength to the theory of  $\beta$ -oxidation.

In the previous paper the fact was emphasized that the position of the double bond is not necessarily the point at which an aliphatic chain is disrupted. This is brought out even more strikingly by the behavior of the two phenylpentenic acids: phenyl- $\beta$ ,  $\gamma$ -pentic acid ( $\text{C}_6\text{H}_5\text{CH}_2\text{CH}=\text{CHCH}_2\text{COOH}$ ) and phenyl- $\alpha$ ,  $\beta$ -pentic acid ( $\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{CH}=\text{CHCOOH}$ ). Both of these acids appear to undergo metabolism in the same way and at the same rate as the saturated phenylvaleric acid itself. The amount of benzoic acid in 24 hours from any one of these three acids was the same and the ratio with which it was conjugated with glycuronic acid and glycine was practically identical for all three acids. Since these three compounds seem to be handled as if

they were one and the same substance, one is led to conclude that the introduction of a double bond into an aliphatic chain, or in other words, the removal of 2 adjacent hydrogen atoms, is a reaction which the body can perform with ease. This is in harmony with the modern theories of tissue oxidation. Since the molecule must be looked upon as a dynamic and not a static entity, it is not surprising that fission should fail to occur at the point of unsaturation. Apparently when the unsaturated aliphatic acid comes under the chemical influences of the body, the unsaturated bond is no longer stationary and may shift, but

TABLE II.

*Fate of Mandelic Acid, Phenyl- $\beta$ -Hydroxypropionic Acid, and Acetophenone When Fed to Dogs.*

Dog 10; weight 8 kilos.

Substance fed.	Amount.	Combined benzoic acid.		Amount of substance excreted unchanged.	Combined phenyl-methylcarbinol.
		With glyceronic acid.	With glycine.		
	gm.	gm.	gm.	gm.	gm.
Phenyl- $\beta$ -hydroxypropionic acid.....	5.0	0.91	0.88	3.75	1.35
“ “ “ “ .....	5.0	0.76	0.62		
Mandelic acid.....	5.0	0.59	0.21		
Acetophenone.....	4.0		0.80		
Blank.....		0.47	0.18		

the point of splitting is determined by other factors. At our present state of knowledge concerning the mechanism of fat metabolism one can merely state that when an aliphatic chain contains a carboxyl group, the carbon atoms which are in  $\alpha$  and  $\beta$  position to this activating group become susceptible to dehydrogenation and subsequent further oxidation, resulting finally into splitting of the chain between these 2 carbon atoms. This of course explains nothing, but is merely a restatement of the hypothesis of  $\beta$ -oxidation in slightly different terms.

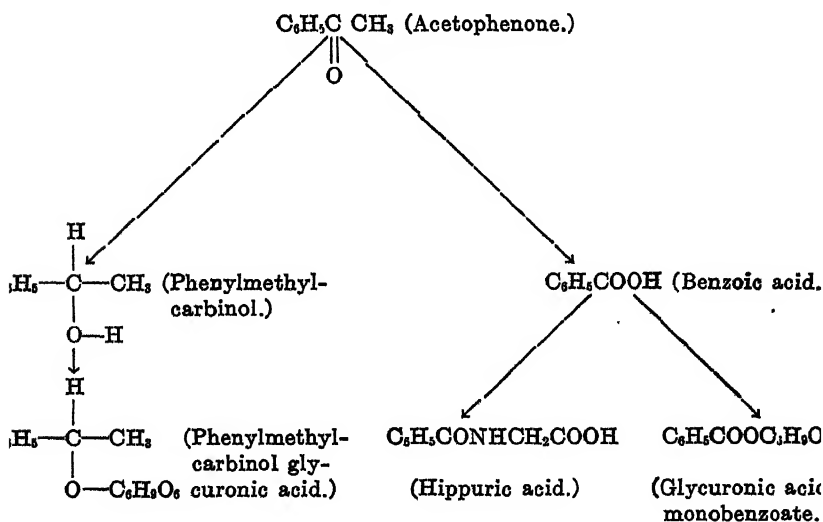
The exact mechanism of  $\beta$ -oxidation is not known, but the original hypothesis of Dakin that one of the first steps is the produc-

tion of the  $\beta$ -hydroxy acid is now open to serious doubt. In fact Dakin (9), in a later article, states that the formation of the hydroxy acid as the first step in the catabolism of fatty acids becomes rather problematical. The difficulty with which the body handles phenyl-substituted hydroxy acids has been observed and commented on by various investigators. Knoop (7) found that mandelic acid when fed to dogs was excreted unchanged. Since Knoop overlooked the conjugation with glycuronic acid in all his studies, the fate of mandelic acid in the body was restudied. As recorded in Table II, it can be seen that the acid is excreted almost entirely unchanged and that it is combined neither with glycine nor with glycuronic acid. Why the introduction of a hydroxy group in the aliphatic chain should cause such a profound change and why mandelic acid is handled so entirely differently from phenylacetic acid, merely indicates the inadequacy of our knowledge concerning the correlation between chemical structure and metabolic processes.

The metabolism of phenyl- $\beta$ -hydroxypropionic acid should offer a means for determining whether the hydroxy acid is an intermediary in the oxidation of phenylpropionic acid. If it is one would expect that it would yield the same end-product, benzoic acid, and that this acid would be conjugated with glycine and glycuronic acid in the same ratio as when derived from phenylpropionic acid itself. An inspection of Table II shows that the metabolism of phenyl- $\beta$ -hydroxypropionic acid is strikingly different from either phenylpropionic acid or cinnamic acid. Approximately 75 per cent of the hydroxy acid is excreted unchanged while the remainder is oxidized to benzoic acid which is conjugated with glycuronic acid and glycine in about equal proportions. These results are so striking that they leave little doubt as to the fact that phenyl- $\beta$ -hydroxypropionic acid is not a normal intermediary product in the catabolism of phenylpropionic acid. The inability of the body to oxidize hydroxy acids is apparently not limited to phenyl- $\beta$ -hydroxypropionic acid. Thus, Dakin (3) found that on injecting phenyl- $\beta$ -hydroxyvaleric acid into animals, a large part of the acid appeared again in the urine unchanged. Knoop and Oeser (10) reported that after feeding 8 gm. of phenyl- $\beta$ -hydroxybutyric acid to a dog, 3.8 gm. appeared again as phenylbutyrolactone, and only 1.3 gm. as phenylaceturic acid. This, it will be

recalled, is very different from the results obtained on phenylbutyric acid which is completely oxidized to phenylacetic acid. If the mechanism of the metabolism of phenyl-substituted aliphatic acids can be considered the same as that of the normal fatty acids, then one can conclude that  $\beta$ -hydroxybutyric acid likewise is not a normal intermediate in the oxidation of butyric acid, but perhaps is formed only in a period of stress such as is encountered when there is an insufficient supply of carbohydrate. Attention should be called to the fact that the benzoic acid which was derived from the oxidation of phenyl- $\beta$ -hydroxypropionic acid is not conjugated in the characteristic 3:1 ratio but approximately in a 1:1 ratio. It is probable that one is again dealing with a delayed production of benzoic acid, consequently the relatively larger production of hippuric acid.

The other compound studied was acetophenone because of its structural resemblance to acetone. In the body, part of the compound is reduced while another part is oxidized. The possible paths of its metabolism can be best presented diagrammatically.



From Table II it can be seen that acetophenone undergoes both oxidation and reduction. The reduced product, phenylmethyl-

carbinol, is conjugated with glycuronic acid in the regular glucoside linkage. The portion that is oxidized to benzoic acid appears in the urine as hippuric acid, but strangely, little, if any, benzoic acid is conjugated with glycuronic acid. Here, we are perhaps again dealing with a very slow production of benzoic acid. The results obtained agree with those of Thierfelder and Daiber (2). They found on feeding acetophenone that 35 per cent was reduced and conjugated with glycuronic acid, and 24 per cent was oxidized to benzoic acid. In the single experiment recorded in this paper, 35 per cent of the acetophenone appeared in the urine as phenyl-methylcarbinol glycuronic acid, while 20 per cent was excreted as hippuric acid. A considerable portion was eliminated unchanged. It might be mentioned that acetophenone is distinctly toxic. On giving 5 gm. to a medium-sized dog, severe convulsions were produced which lasted for an hour. When the absorption was slowed by mixing the drug with food, the same dose failed to produce convulsions or other untoward symptoms.

Unfortunately, the metabolism of benzoylacetic acid was not investigated, but judging from the results obtained by Friedmann (11) it seems rather definite that the compound like phenyl- $\beta$ -hydroxypropionic acid fails to be metabolized like phenylpropionic acid. Friedmann found that only 13 per cent of the acid was excreted as hippuric acid, and that phenyl- $\beta$ -hydroxypropionic acid, acetophenone, and cinnamoyl glycoll were formed and excreted. Thus, on analyzing the results obtained in the present study and those obtained by the various investigators who have studied phenyl aliphatic acids, one finds that there is considerable evidence against the assumption that the mechanism of  $\beta$ -oxidation proceeds through the hydroxy and the ketonic acid stage. The fact that the unsaturated acids of the phenyl aliphatic series containing 3, 4, and 5 carbon atoms are handled exactly like their corresponding saturated acids, while both the hydroxy acids and the ketonic acids appear to be handled by a different type of metabolism as indicated by their excretory end-products, leads one to conclude that the unsaturated acids are regular intermediary compounds in the metabolism of the phenyl-substituted aliphatic acids, whereas the hydroxy and ketonic acids are not. Because of the analogy between the phenyl aliphatic acids and the normal fatty acids, these conclusions may be of

significance. It must be remembered that there is no direct proof that  $\beta$ -hydroxybutyric acid and acetoacetic acid are normal intermediates in the catabolism of butyric acid. If it is permissible to interpret the results obtained on the phenyl aliphatic acids in terms of normal fatty acids, it appears doubtful whether the so called acetone bodies are formed even temporarily in the normal metabolism of butyric acid. It is quite possible that their formation may represent an entirely abnormal metabolic path.

## SUMMARY.

1. Phenylvaleric acid, phenyl- $\alpha$ ,  $\beta$ -pentenic acid, and phenyl- $\beta$ ,  $\gamma$ -pentenic acid when fed to dogs yield only benzoic acid, which is conjugated with glucuronic acid and glycine in the ratio of 2:1. The similarity of these results with those obtained on phenylpropionic acid, cinnamic acid, and benzoic acid itself, lends further support for the theory of  $\beta$ -oxidation.

2. Mandelic acid is neither oxidized nor conjugated by the dog, but is excreted unchanged.

3. Phenyl- $\beta$ -hydroxypropionic acid is excreted largely unchanged, but about 25 per cent is oxidized to benzoic acid which is conjugated with glucuronic acid and glycine in the ratio of about 1:1.

4. Acetophenone, when given *per os* is partially reduced to phenylmethylcarbinol which appears conjugated with glucuronic acid. Another portion is oxidized to benzoic acid which is excreted entirely as hippuric acid.

5. The metabolism of phenyl- $\beta$ -hydroxypropionic acid and acetophenone as indicated by their excretory end-products is distinctly different from the metabolism of phenylpropionic acid. Therefore neither phenyl- $\beta$ -hydroxypropionic acid nor acetophenone appears to be a normal intermediate in the catabolism of phenylpropionic acid.

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## QUANTITATIVE STUDIES OF $\beta$ -OXIDATION.

### III. THE FATE OF PHENYLBUTYRIC ACID IN DEPANCREATIZED DOGS.\*

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Among the many unanswered questions that arise in the consideration of the relationship between the metabolism of fats and carbohydrates there is one which is of particular interest; namely, "Can the completely diabetic organism still oxidize butyric acid to carbon dioxide and water?" Stated more specifically, "Is any butyric acid burned completely in the total absence of carbohydrate metabolism?" It is a well known fact that if for any reason carbohydrate metabolism is depressed or fat metabolism unduly increased, ketonuria results. Thus has originated that vague and almost meaningless expression that fats are burned in the fire of carbohydrates. The well known work of Shaffer clearly demonstrates that there is a quantitative relationship between the metabolism of fatty acids and carbohydrates. In spite of the practical usefulness of Shaffer's theory it throws very little light on the actual mechanism by which the presence of carbohydrates aids in the combustion of fatty acids. It seems highly probable that a true union or chemical reaction occurs between glucose or one of its derivatives and the fatty acid at some stage of its catabolism. What the actual chemistry is, remains unknown, but various theories have been proposed each recognizing that the crucial point in the catabolism of the fatty acid is at the butyric acid stage. Shaffer (1) himself from experiments *in vitro* in which he found that the oxidation of acetoacetic acid by hydrogen peroxide is accelerated in the presence of glucose has suggested that a chemi-

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

cal union between glucose and acetoacetic acid takes place in the body. In gathering support for this theory from a purely chemical point of view, West (2) has studied and prepared various condensation products of glucose and acetoacetic acid. Ringer (3) at one time proposed that glucose might combine with  $\beta$ -hydroxybutyric acid in glucosidic linkage. Woodyatt (4) suggested the possibility of glyceric aldehyde combining with acetoacetic acid. Geelmuyden (5) called attention to the probability that acetone bodies may be conjugated with carbohydrates in a manner similar to the glycuronic acid type of conjugation. He referred, of course, to the glucosidic class of glycuronic acid linkage. In consideration of all these theories, it should be recognized that there is no conclusive proof that any one of the acetone bodies is a normal intermediate in the metabolism of fatty acids. One of us (6-8) has mentioned repeatedly in previous papers the possibility of a conjugation between the fatty acid and glucose or one of its derivatives forming a compound very similar in type to the one produced when benzoic acid or phenylacetic acid is conjugated with glycuronic acid.

If a chemical union between the fatty acid and glucose is necessary for the complete combustion of the former, it can be concluded that if carbohydrate metabolism could be entirely abolished all of the fat would be excreted in the form of acetone bodies. The nearest one can approach an absolute cessation of carbohydrate metabolism is in complete pancreatic diabetes, but even in this condition, as will be brought out later, one does not know whether a part of the potential sugar in the protein molecule may not be available and utilized by the organism. Various quantitative studies have, of course, been made to determine how much of the fat in diabetes appears in the form of acetone bodies. It is obviously not possible to review critically all these experiments, but it can be inferred that they do not answer conclusively the question whether butyric acid is excreted quantitatively in the form of acetone bodies when metabolized supposedly in the total absence of insulin. Thus Leathes and Raper (9) conclude: "Each molecule of fatty acid undergoing catabolism has not been proved to give rise to 1 molecule of acetoacetic acid as the theory would require, but to a smaller amount."

It is exceedingly difficult to determine by the ordinary metabolic

experiments whether or not any butyric acid is burned in diabetes. The conclusions must necessarily be based on indirect evidence, and on calculations depending on such assumptions as that each molecule of fatty acid yields 1 molecule of butyric acid, and that the amount of sugar derived from protein is fixed and constant. Even such reliable factors as the respiratory quotient can give rise to serious errors of interpretation unless carefully controlled.

Obviously, it is hardly to be expected that the standardized metabolic experiments can solve the problem whether the diabetic organism can oxidize, at least in part, butyric acid to carbon dioxide and water. It seemed that a partial solution of the problem might be obtained by studying the fate of phenylbutyric acid in a depancreatized dog, since if the compound is oxidized under those conditions, its end-products can be isolated and identified. Thus, if after feeding phenylbutyric acid to a diabetic dog, phenylacetic acid is found in the urine, it would show that the phenyl-substituted butyric acid can be burned. One would be inclined to conclude that normal butyric acid likewise is burned in complete diabetes. One must not forget, however, that it is not possible to state to what extent the metabolism of fatty acid is comparable to an aliphatic acid having a phenyl group replacing a hydrogen atom on the distal carbon atom. The various experiments in which these acids have been employed for the study of  $\beta$ -oxidation seem to indicate that their metabolism is similar to that of the normal fatty acids. Moreover, it is safe to infer that if the organism is able to oxidize a fatty acid containing a phenyl group, it ought to have even less trouble with the normal unsubstituted chain of carbon atoms of the fatty acid.

In this work it was found that the depancreatized fasting dog even in the terminal stages was able to oxidize phenylbutyric acid to phenylacetic acid, which was isolated from the urine as phenylaceturic acid and definitely identified. If one accepts the supposition that the phenyl-substituted fatty acids are metabolized by the same mechanism as the normal fatty acids, then it follows that butyric acid likewise can be oxidized at least to some extent in the diabetic animal. The experiments were performed on dogs, and it is a recognized fact that this animal is resistant to ketonuria and the accompanying acidosis. It is well to bear in mind how-

ever that even in man a certain resistance to ketosis can be presumably acquired, for Folin and Denis (10) observed that after repeated fasting, obese subjects developed the ability to fast without producing a ketonuria.

Since the experiments reported in this paper suggest that a small amount of butyric acid may be completely burned in the diabetic animal, it seems probable that perhaps a portion of the protein molecule may still act as a ketolytic substance in total pancreatic diabetes. It should be recalled that the depancreatized dog can still conjugate benzoic acid with glycuronic acid and that the latter compound is derived from the glycogenic fraction of the body proteins (11).

Due to the difficulty in making the diabetic dog retain phenylbutyric acid when given *per os*, it was not possible to get as satisfactory quantitative results as those reported previously on normal dogs. Since the feeding of phenylbutyric acid caused both vomiting and diarrhea, the amount retained could not be determined. It was possible however to establish definitely that phenylbutyric acid is burned to phenylacetic acid in the depancreatized dog. The phenylacetic acid thus formed is excreted in part as phenylaceturic acid which can readily be isolated and identified. It is highly probable that some of the phenylacetic acid is also conjugated with glycuronic acid, but since that determination depends on an indirect method, it cannot be considered as conclusive as the actual isolation of phenylaceturic acid. Whether any of the phenylbutyric acid is converted to phenyl- $\beta$ -hydroxybutyric acid, benzoylactic acid, or acetophenone, is difficult to state since none of these compounds was isolated from the urine. There is some indication that perhaps some acetophenone is formed. On inspecting Table I it will be observed that the urine collected after the feeding of phenylbutyric acid had a distinctly lower optical rotation than the calculated value based on the amount of glucose present, and further that the urine after extraction with ether had a higher positive optical rotation. This indicates that the urine contained a levorotatory substance which was at least partially soluble in ether. Whether this substance was phenylmethylcarbinol glycuronic acid, one of the main excretory products when acetophenone is fed, was not determined.

TABLE I.

Dog 1; weight 7.0 kilos. Operated upon April 18; no food or insulin after April 23.

Date.	Sugar.	Nitrogen.	D:N	Acetone.	Combined phenylacetic acid.		Remarks.
					With glyconic acid.	With glycine.	
1928	gm.	gm.			gm.	gm.	
Apr. 24	29.2	4.28	6.80				
" 25	20.1	5.32	3.76	+			
" 26	14.0	6.01	2.32	++++			
" 27	7.8	4.85	1.61	++++		0.82	8.5 gm. phenylbutyric acid fed.*
" 28†	4.4‡	4.05	1.10	++	0.25	0.77§	4.0 gm. phenylbutyric acid fed.

\* The dog vomited after being fed 5.0 gm. of phenylbutyric acid, and most of the material was lost. An hour later, 3.5 gm. more of phenylbutyric acid were given. Since both vomiting and diarrhea set in, the amount of material retained could not be determined.

† Attempts to restore the dog to a normal metabolism with the aid of insulin and food failed, and the dog died 1 day after the close of the experiment.

‡ Urine volume, 300 cc.  $\alpha$  observed in a 1 dm. tube,  $+0.35^\circ$ ; after extraction with ether,  $+0.55^\circ$ .

§ Phenylaceturic acid was definitely identified by a melting point determination.

|| The neutralized acid was fed in 1 gm. doses every half hour until 4.0 gm. had been given. The dog vomited once but the vomitus was refed.

TABLE II.

Dog 2; weight 8.0 kilos. Operated upon May 9; no food or insulin after May 14.

Date.	Sugar.	Nitrogen.	D:N	Acetone.	Combined phenylacetic acid.		Remarks.
					With glyconic acid.	With glycine.	
1928	gm.	gm.			gm.	gm.	
May 15	11.8	3.76	3.12	+			
" 16	13.7	5.60	2.44	++++			
" 17	9.9	4.90	2.05	++++	1.04	0.89*	5.0 gm. phenylbutyric acid fed.†

\* Phenylaceturic acid definitely identified by a melting point determination.

† Dog fed 2.5 gm. of phenylbutyric acid. 50 minutes later the dog had watery diarrhea. The second 2.5 gm. dose was retained.

## EXPERIMENTAL.

Female dogs were employed. After pancreatectomy the animals were kept on a diet of meat and sucrose to which a little pancreatin had been added. Insulin was given twice a day, the total dose being 20 to 30 units. After the animal had recovered from the operation, both insulin and food were withdrawn. After 3 to 5 days of fasting, phenylbutyric acid neutralized with a calculated amount of sodium hydroxide and mixed with a 1 per cent agar solution was given by means of a stomach tube. Serious difficulties were encountered, for not only did the animal vomit, but it also developed a violent diarrhea which set in as early as 30 minutes after feeding. It was a rather puzzling problem to overcome this difficulty, for a severely diabetic dog is a very sick animal, and a gastrointestinal upset can be induced with trigger-like rapidity. Since it was found that the small amount of impurities present in phenylbutyric acid was more irritating than the acid itself, better results were obtained when a carefully purified product was used. The other means for controlling the vomiting and diarrhea consisted in giving small divided doses instead of a large single dose.

*Analytical Methods.*

The quantitative procedures used were essentially the same as those described in the earlier papers. Glucose was determined by the Shaffer and Hartmann macro method. Phenylaceturic acid was determined by the method previously described for the quantitative estimation of hippuric acid (12).

*Glycuronic Acid Monophenylacetate.*—A measured volume of urine was acidified with dilute sulfuric acid until acid to Congo red. The acidified urine was extracted in 10 cc. portions with ether in the continuous type of extractor previously described (12). In this procedure phenylaceturic acid and free phenylacetic acid are completely removed, while glycuronic acid monophenylacetate, which is relatively insoluble in ether, is left in the urine. The extracted portions of urine were united and treated with alkali until neutral to litmus. To 50 cc. of the urine treated in this manner, 1 gm. of sodium carbonate was added and the solution boiled for 1 minute. Under those conditions glycuronic acid

monophenylacetate is completely hydrolyzed. The urine was reacidified and the liberated phenylacetic acid extracted with toluene, and determined by titrating the toluene solution with standardized sodium alcoholate. The actual details of the procedure were the same as those described by Raiziss and Dubin (13) for determining free benzoic acid. From the amount of phenylacetic acid found, the quantity of glycuronic acid monophenylacetate can readily be calculated.

*Identification of Phenylaceturic Acid.*—Acidified samples of urine were extracted with ether in a continuous extractor, the ether evaporated off, and the crystalline residue purified by recrystallization from hot water, a small amount of decolorizing charcoal being used. The melting point of the phenylaceturic acid thus obtained agreed with the value of  $143^{\circ}$  found in the literature. Besides this exact method for identifying phenylaceturic acid, there are several simple methods for recognizing this compound and differentiating it from hippuric acid. Thus, if the syrupy extract from which the ether has been driven off be inoculated with a tiny crystal of phenylaceturic acid immediate crystallization will occur if the extract contains phenylaceturic acid whereas nothing happens if only hippuric acid is present. If the extract containing either phenylacetic acid or hippuric acid is allowed to crystallize spontaneously it is possible by inspection to differentiate these two compounds since the crystalline structures both microscopically and macroscopically are markedly different. The phenylaceturic acid isolated from the urine of depancreatized dogs after being fed phenylbutyric acid was definitely identified by the tests described.

#### SUMMARY.

The depancreatized dog retains the power to oxidize phenylbutyric acid to phenylacetic acid and to conjugate the latter with glycine and with glycuronic acid. Because of the relatively close relationship between the phenyl aliphatic acids and the normal fatty acids both as to their chemical structure and their physiological behavior, these findings suggest that the diabetic organism probably can still oxidize completely a small amount of butyric acid.

The theory is proposed that in the metabolism of fatty acids, butyric acid or one of its metabolic derivatives is chemically combined with a carbohydrate group similar in type to the conjugation of benzoic acid with glycuronic acid.

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## QUANTITATIVE STUDIES OF $\beta$ -OXIDATION.

### IV. THE METABOLISM OF CONJUGATED GLYCURONIC ACIDS.\*

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The position and importance of glycuronic acid in the intermediary metabolism of glucose is at best but imperfectly understood even though it has been the subject of repeated investigations. No direct proof can be found that glycuronic acid is burned in the body, but there is fairly good evidence that it can be metabolized at least in small amounts. This is indicated, for instance, by the work of Hürthle (1), whose paper is one of the most recent contributions on the subject of the metabolism of glycuronic acid. On injecting sodium glycuronate into rabbits and dogs, he found that a portion of the material was excreted in the urine, but the amount which he recovered fell far short of the quantity which he had administered. A series of preliminary and unpublished experiments by the author gave similar results. On ingesting 3 gm. of neutralized glycuronic acid, he could detect no reducing substance in the urine with Benedict's reagent, but when he took larger amounts (10 gm.) a part of the glycuronic acid appeared in the urine. In the light of these observations as well as those of Hürthle (1) and of the earlier work of Biberfeld (2), one is led to conclude that the capacity of the organism to burn glycuronic acid is rather limited, and that the contentions of Mayer (3), who has attempted to prove that glycuronic acid is readily metabolized, are untenable. Likewise the isolated experiment of Baumgarten (4) who could find no non-fermenting reducing substance in the urine after feeding a diabetic patient 13.5 gm. of glycuronic acid lactone, is in direct contradiction to

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

the author's observations on normal subjects, and it is highly improbable that the diabetic organism has acquired metabolic powers not possessed by the normal.

The difficulty that the body encounters in metabolizing glycuronic acid shows quite clearly the profound change that occurs in the glucose molecule when a carboxyl group is introduced on the terminal carbon atom. This alteration in physiological properties is further illustrated by the relative effects which glucose and glycuronic acid have on insulin convulsions. While glucose promptly abolishes the insulin shock and restores the animal to the normal state, glycuronic acid exerts no demonstrable beneficial effects. Experiments in this connection were carried out upon seven mice by the same method and procedure as employed by Herring, Irvine, and Macleod (5). In no case was an animal suffering from insulin convulsions benefited through administration of 5 or 10 drop doses of a 15 per cent solution of glycuronic acid.

The author's work was carried out over 2 years ago, and during this interval Hürthle (1) has reported a similar experiment. He found that a rabbit in insulin convulsions was not relieved by glycuronic acid. The physiological inertness of glycuronic acid is significant because it does throw some light on the relationship of the structure of glucose to its metabolism. There is an instinctive tendency to consider the aldehyde group of a sugar the most susceptible to reaction, and the portion of the chain nearest to this group, the part of the molecule which is most involved in the initial chemical reactions that occur when the sugar is metabolized. But the fact that glycuronic acid is produced with such ease by the body, and when once formed, metabolized with difficulty, suggests that the terminal alcoholic group of glucose may perhaps be fundamentally involved in the initial chemical changes occurring during metabolism. Although glycuronic acid is probably not a normal intermediate in the combustion of glucose, it may perhaps become the key to important knowledge concerning the chemistry underlying the metabolism of carbohydrates.

Since glycuronic acid in the animal is always encountered in a conjugated form it is rather surprising to find that practically nothing is known about the metabolism of combined glycuronic

acid. In fact as far as the writer is aware this problem has not been investigated heretofore. Information concerning the behavior of conjugated glycuronic acids in the body should be useful, for it ought to enable one to interpret more intelligently many of the experiments designed to study the production of glycuronic acid. There are two known classes of conjugated glycuronic acids: the glucosidic type, of which menthol glycuronic acid is a good example, and the ester type, of which glycuronic acid monobenzoate is the outstanding and most important member. Since there is considerable difference between these two classes of compounds, it is best to discuss them separately.

With the exception of a few recent papers on glycuronic acid monobenzoate, practically all of the previous studies on glycuronic acid deal with the glucosidic type. As there was no simple and reliable quantitative method for determining conjugated glycuronic acids prior to the author's method for menthol glycuronic acid (6), most of the earlier work was qualitative. It is easy to understand therefore why no attention was paid to the possible metabolism of combined glycuronic acids. In a quantitative study of the production of menthol glycuronic acid in rabbits, the author (7) found that less than 50 per cent of the menthol fed appeared in the urine and that only a trace of conjugated menthol was present in the feces. These results suggest that the rabbit possesses the power to oxidize or destroy menthol, and therefore the amount of menthol glycuronic acid excreted is not an index of the animal's power to synthesize glycuronic acid, but rather a measure of its ability to metabolize menthol. When the study of the production of menthol glycuronic acid was extended to dogs, the interesting discovery was made that only a very small portion of the menthol fed was excreted in the urine with glycuronic acid. Equally remarkable were the results obtained when menthol glycuronic acid was fed to dogs, for again only a small amount of the material appeared in the urine and only a trace in the feces. Apparently, the dog has a marked power to oxidize not only menthol but also menthol glycuronic acid. The rather perplexing question arises whether all of the menthol is first conjugated with glycuronic acid and then burned, or whether some of the menthol is oxidized directly. In other words, it raises the question whether in the catabolism of phenolic compounds a preliminary conjuga-

tion with glycuronic acid is necessary. Borneol, like menthol, when fed to dogs, is partly destroyed, for the amount of borneol glycuronic acid appearing in the urine accounts for only about 50 per cent of the amount administered. Similarly, when borneol glycuronic acid is fed, a considerable fraction fails to be excreted, although the destruction of either borneol or borneol glycuronic acid is far less complete than that of menthol and menthol glycuronic acid (Table I). In man the percentage of menthol and borneol conjugated with glycuronic acid is higher than either in dogs or rabbits. It is very probable that many other conjugated

TABLE I.  
*Metabolism of Glucosidic Types of Conjugated Glycuronic Acids.*

Subject.	Compound fed.	Amount.	Equiv- alent in terms of borneol or men- thol.	Borneol or menthol con- jugated with glycuronic acid.	
				gm.	per cent
Dog.....	Borneol.	5.0	5.00	2.60	52
" .....	" glycuronic acid.	5.0	2.34	1.59	68
" .....	Menthol.	5.0	5.00	0.26	5
" .....	" glycuronic acid.	5.0	2.32	0.32	14
Man.....	Borneol.	2.0	2.00	1.63*	81
" .....	"	3.5	3.50	2.41†	69
" .....	Menthol.	1.0	1.00	0.79†	79

\* In 10 hours.

† In 6 hours.

glycuronic acids, especially phenol glycuronic acid, are destroyed in part by the body. Since the quantity of conjugated glycuronic acid present in the urine is not necessarily an index of the organism's power to synthesize glycuronic acid, but rather a measure of the inability of the body to oxidize the phenolic compound administered, the interpretations of many experiments on glycuronic acid may have to be revised or altered. The literature is too extensive to review comprehensively all the studies made on glycuronic acid, but several recent papers will illustrate the foregoing statement. Thus, the findings of Sister Mary Adeline (8) that the output of glycuronic acid, as measured by the amount of

menthol glycuronic acid excreted, is increased when certain amino acids are fed, does not prove that these amino acids have any influence on the synthesis of glycuronic acid. It is much more likely that these substances may have a stimulating or diuretic effect causing a greater excretion of menthol glycuronic acid with a corresponding decrease in the fraction undergoing complete destruction. Similarly the observations of Friedemann and Koechig (9) that the production of menthol glycuronic acid is diminished after the administration of insulin and of phlorhizin are utterly

TABLE II.  
*Fate of Glycuronic Acid Monobenzoate in Man.*

Time.	Benzoic acid excreted as hippuric acid.*			Remarks.
	Total.	Total—blank.†	Net amount per hr.	
	gm.	gm.	gm.	
9 a.m.—12 n.	0.13		0.04	1 cup coffee and 1 doughnut at 9.00 a.m.
12 n. — 2 p.m.	0.28	0.20	0.10	5.0 gm. neutralized glycuronic acid monobenzoate at 12.00 n.
2 — 3 "	0.27	0.23	0.23	
3 — 6 "	0.78	0.66	0.33	2 boiled eggs at 6.00 p.m.
6 — 10 "	0.69	0.53	0.14	
10 p.m.— 5 a.m.	0.29	0.01	0.00	

\* The urine at no time showed reduction with Benedict's solution.

† The net amount of benzoic acid excreted in 20 hours was 1.63 gm. The quantity of benzoic acid in 5 gm. of glycuronic acid monobenzoate is 2.05 gm.

worthless as evidence that these drugs have any influence on the synthesis of glycuronic acid. These results merely show how much menthol and menthol glycuronic acid escape oxidation and not how much glycuronic acid is actually synthesized. It should be recalled at this point that in studying the production of glycuronic acid in depancreatized dogs following the feeding of benzoic acid, no diminution of the animals' power to synthesize glycuronic acid monobenzoate was observed (10).

The study of the metabolism of glycuronic acid monobenzoate as recorded in Tables II and III indicates definitely that the glycu-

ronic acid portion of the molecule can be and is oxidized by the animal organism. When glycuronic acid monobenzoate is fed to man none of it appears in the urine, but the benzoic acid is excreted in the form of hippuric acid. If glycuronic acid monobenzoate is given to dogs, a relatively large portion appears in the urine unchanged, and the remaining benzoic acid is eliminated as hippuric acid. It is important to note that when glycuronic acid monobenzoate is administered parenterally, the ratio of the quantity excreted unchanged to that of hippuric acid is roughly the same as when benzoic acid is fed directly. This suggests the interesting possibility that all of the benzoic acid may perhaps be first conjugated

TABLE III.  
*Fate of Glycuronic Acid Monobenzoate in Dog.*

Compound fed.	Amount.	Equivalent in terms of benzoic acid.	Combined benzoic acid.					
			With glycuronic acid.	With glycine.	Corrected for blank.			
					With glycuronic acid.		With glycine.	
	gm.	gm.	gm.	gm.	gm.	per cent	gm.	per cent
Glycuronic acid monobenzoate.....	2.0	0.82	0.41	0.42	0.24	39	0.38	61
" " " *	1.0	0.41	0.39	0.15	0.22	67	0.11	33
Benzoic acid.....	1.0	1.00	0.61	0.41	0.44	54	0.37	46
Blank.....			0.17	0.04				

\* Given subcutaneously.

with glycuronic acid, and that the hippuric acid is produced only secondarily from the glycuronic acid compound. Such at least appears probable in the case of the dog. Whether the same be true in humans remains to be established by further experiments. The facts, first, that glycuronic acid monobenzoate appears to be the primary compound formed when benzoic acid enters the body, and second, that the body can oxidize the glycuronic acid attached to the benzoic acid, add further strength to the theory that the metabolism of butyric acid may involve a conjugation with a carbohydrate group similar in type to the benzoic acid-glycuronic acid complex (11).

## SUMMARY.

Glycuronic acid does not cure or alleviate insulin convulsions in mice.

When either menthol or menthol glycuronic acid is fed to dogs only a small amount of conjugated menthol appears in the urine, thus indicating that the organism has the power to oxidize both menthol and conjugated glycuronic acid. Borneol and borneol glycuronic acid are similarly destroyed but to a less extent. It is probable that other conjugated glycuronic acids of the glucosidic type are similarly oxidized. Since the amount of menthol or borneol glycuronic acid appearing in the urine is a measure merely of the amount of menthol or borneol which has escaped oxidation rather than an index of the power of the organism to synthesize glycuronic acid, the conclusions of many of the older investigators probably are erroneous.

When glycuronic acid monobenzoate is ingested by man, only hippuric acid appears in the urine. Glycuronic acid monobenzoate given to dogs either by mouth or parenterally is in part excreted unchanged and in part apparently oxidized since a portion of the benzoic acid is eliminated as hippuric acid. These results suggest the possibility that in the dog all the benzoic acid is first conjugated with glycuronic acid, and that hippuric acid is formed secondarily from glycuronic acid monobenzoate.

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# THE FURTHER APPLICATION OF THE VANILLIN-HYDRO- CHLORIC ACID REACTION IN THE DETERMINATION OF TRYPTOPHANE IN PROTEINS.

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In a previous paper (1) it was shown that the vanillin-hydrochloric acid reaction for tryptophane is more sensitive than other color reactions used previously, that the reaction is more specific in distinguishing tryptophane from some of its decomposition products, and that it can be used in the quantitative recovery of tryptophane from amino acid mixtures comparable to the composition of casein. The accuracy of the method being established it was possible to apply it upon the mixtures obtained by various methods of hydrolysis of proteins. The results clearly show that basic hydrolysis (by barium hydroxide) of proteins by the procedure employed by Folin and Looney (2) actually causes a loss of tryptophane as high as 20 per cent. The Folin-Looney procedure was shown to introduce an error since it neglects the volume occupied by the barium sulfate precipitate formed in removal of the barium after the hydrolysis. It was also shown that even hydrolysis by alkaline trypsin solution leads to some loss of tryptophane and that in this destruction three factors are involved; namely, incubation, trypsin, and the diamino acid fraction from proteins. Omission of one of these three factors does not lead to loss of tryptophane.

Looney (3) has attacked this paper and aims to discredit the findings and conclusions. It is, however, quite clear that he has not read the paper sufficiently carefully to warrant his generalized conclusions. He, as well as Onslow (4) whom he cites as evidence in his favor, admits that tryptophane is decomposed in part by barium hydroxide hydrolysis. He assumes, however, that the

products of decomposition are of such nature that they react, molecule for molecule, in exactly the same manner with the Folin-Looney reagent as to color intensity, as to the tryptophane lost and that therefore the Folin-Looney method is reliable. He does not prove his point on amino acid mixtures of known content as to tryptophane.

*I. Slight Modification of Procedure in Vanillin-HCl Reaction to Save Time and Material.*

The precipitation of tryptophane, washing, *etc.*, are carried out exactly as described in the previous publication (1) but instead of the precipitate being transferred by means of concentrated hydrochloric acid into a volumetric flask, the reaction is allowed to take place directly in the centrifuge tube. Thus, to the 15 cc. centrifuge tube containing the washed mercury-tryptophane precipitate add 1 cc. of 2 per cent mercuric sulfate solution (found to intensify color), 0.4 cc. of 0.5 per cent vanillin reagent, and 12 cc. of concentrated hydrochloric acid. The acid is added gradually while the precipitate is mixed with a stirring rod. The precipitate dissolves, and the stirring rod is rinsed with the remaining acid. The tube is stoppered with a cork and allowed to stand 24 hours, then compared in the colorimeter. In the previous work a standard of 0.4 mg. was used; throughout this work a 0.2 mg. standard was used. Tryptophane in concentrations of less than 0.2 mg. cannot be precipitated quantitatively under the conditions described. Therefore, in cases where only traces of tryptophane are indicated, such as in blanks, *etc.*, known amounts of tryptophane are added (usually the same amount as in the standard) so as to insure quantitative precipitation.

*II. Application of Method on Highly Purified Proteins Directly and on Trypsin Hydrolysates of the Same Protein.*

The following estimations were made on each protein.

1. *Direct Vanillin-HCl Reaction.*—0.1 gm. of each of the proteins listed below was weighed directly into the 15 cc. centrifuge tube to which the vanillin reagent and the concentrated hydrochloric acid were added and allowed to stand for 24 hours.

2. *Indirect Vanillin-HCl Reaction.*—0.5 gm. of protein and 0.25 gm. of U.S.P. pancreatin were weighed into small Erlenmeyer

flasks containing 25 cc. of 0.4 per cent  $\text{Na}_2\text{CO}_3$ , preserved with toluene, and incubated for 5 days at  $37^\circ$ . An aliquot portion was then used for the reaction. In some instances the protein hydrolysate after acidification had to be run through the quantitative filter several times before it filtered clear. It was found that the addition of a very small amount of fullers' earth greatly facilitated

TABLE I.

Protein.	Per cent tryptophane.		
	By Jones.*	By direct vanillin-HCl.	By indirect vanillin-HCl.
Gliadin (wheat)†.....	0.71	Trace (reddish).	0.60
Speltn (spelt)†.....	1.08	" "	0.74
Dicocumin ( <i>Triticum dicoccum</i> )†	0.80	" "	0.97
Monococumin (einkorn)†.....	0.48	" "	0.47
Secalin (rye)†.....	0.36	Reddish tint.	0.52
Sativin (oats)†.....		0	0.13
Teozein†.....		0	0.19
Kafirin†.....	0.73	Trace (reddish).	0.79
Hordein (barley)†.....	0.45	" "	0.60
Castor bean†.....		Reddish.	1.67
Flaxseed†.....		" blue.	0.90
Excelsin†.....			1.48
Squash seed†.....		Reddish.	1.89
Casein.....		"	1.23
Edestin.....		Bluish.	1.27
Witte's peptone.....		Reddish.	2.97

\* Jones, D. B., cited in Colloid symposium monograph, New York, 1925, ii, 317.

The writer wishes to thank Dr. R. A. Gortner for small samples of the proteins marked †, and Dr. Felix Saunders for those marked ‡. Pfanstiehl casein and Witte's peptone were used. Edestin was prepared from hemp seed.

obtaining a clear filtrate. In Table I are given the results obtained. The figures given in the last column are the average values obtained by two different procedures of the indirect method. The one procedure was the regular method as here modified and the other was the same with the exception that a smaller portion (1 cc.) of the filtrate was employed and 0.2 mg. of tryptophane was added to aid in the mercuric sulfate precipitation of traces, if present. After deduction of the 0.2 mg. added, the results ob-

tained by these two procedures were well within experimental error. In every case the amount of tryptophane in the pancreatin was deducted.

The results in Table I confirm the previous findings that the direct vanillin-HCl reaction cannot be used on proteins directly. What is true for the direct vanillin-HCl reaction is true for any colorimetric method if pure tryptophane is used as a standard. Other workers have used casein as a standard, the unreliability of which is self-evident from the fact that their assumed concentration of tryptophane in casein varies; thus, May and Rose (5) assume 1.5 per cent, Fürth (6) 1.7 per cent, Jones, Gersdorff, and Moeller (7) 2.2 per cent. The percentages obtained by the indirect vanillin-HCl reaction on tryptic hydrolysate are slightly higher than those reported by Jones with the exception of gliadin and speltin. Sativin and teozein, reported negative, are shown to contain a small amount, comparable to gelatin or zein.

In order to ascertain whether any tryptophane is left in the filtrate from the mercuric sulfate precipitation, the following experiment was carried out. 15 gm. of Pfanstiehl casein were hydrolyzed by U.S.P. pancreatin in the usual manner and the tryptophane precipitation carried out as directed in the indirect vanillin-HCl reaction. The filtrate or the clear centrifugalized solution and washings from the mercuric tryptophane precipitate were treated with  $H_2S$  to remove excess of mercury, filtered, and washed. To the combined filtrate and washings barium hydroxide was added to just remove the sulfate ions. The heavy barium sulfate precipitate was thoroughly washed with hot water until a negative Ehrlich diazo test for histidine was obtained. (The diazo test is sensitive and when negative it is safe to conclude that all amino acids have been washed away.) The neutral bulky solution was concentrated *in vacuo* so that 25 cc.  $\approx$  to 5 gm. of original protein. The Hopkins-Cole reaction was applied qualitatively and instead of a tryptophane test, a heavy reddish brown ring comparable to a Molisch test was obtained. Even when a few drops of tryptophane solution were added the Molisch test color obscured the characteristic tryptophane ring. When the solution was diluted no ring was obtained with the Hopkins-Cole reagent. The indirect vanillin-HCl test was next applied. The results are given in Table II.

In the undiluted solution some interfering substance is precipitated, whereas in the diluted solution there is definitely no indication of tryptophane, thus showing that the precipitation was complete.

There is one other possibility that the figures given do not represent the exact concentration of tryptophane in the protein molecule, and that is that possibly tryptophane exists in peptide form which may react differently with the vanillin reagent than the free amino acid. Hunter and Borsook (8) state that glycyl-tryptophane with formaldehyde gives a more reddish color than free tryptophane. The mercury-tryptophane precipitate from a U.S.P. pancreatin hydrolysate of casein contains peptide nitrogen,

TABLE II.

Tube No.....	1	2	3	4	Stand- ard.
	cc.	cc.	cc.	cc.	cc.
Above solution.....	1	1.0			0
“ “ diluted 1:5.			1	1.0	0
Tryptophane 0.05 per cent	0	0.4	0	0.4	0.4
H <sub>2</sub> O.....	1	0.6	1	0.6	1.6
Proceeded in the usual manner.....					
Colorimetric readings.....	No color.	17 mm. Reddish.	No color.	15.1 mm. 15.2 “	15 mm.

the composition of which is being investigated. However, if the precipitate is decomposed and then redigested with trypsin (or U.S.P. pancreatin), or, if the trypsin hydrolysis is followed by the action of erepsin for 48 hours and then the tryptophane precipitated, no peptide nitrogen is found in the precipitate. The tryptophane concentration remains unchanged. This shows that either tryptophane is not a part of the peptide in the mercury-tryptophane precipitate, or if it is, it reacts with the vanillin reagent, giving the same depth and tint of color as the free amino acid.

The work of Komm (9) on the influence of proline and proteins containing proline, especially gelatin, in hastening the color formation of the tryptophane aldehyde reactions, was repeated and confirmed, with the same aldehydes he used; *i.e.*, formaldehyde and *p*-dimethylaminobenzaldehyde. However, when the same prep-

arations and concentrations of proline, hydrolyzed gelatin, and unhydrolyzed gelatin were added to the vanillin-HCl tryptophane reaction the following results were obtained:

4.5 mg. proline.....	No effect.
3.5 " unhydrolyzed gelatin.....	" "
17.0 " hydrolyzed " .....	reddish tint, color comparison cannot be made.

If higher concentrations of unhydrolyzed gelatin are used, a similar secondary reddish color appears. Thus the so called proline effect, by means of which the concentration of proline or "pyrrole rings" in the protein molecule is calculated, cannot be obtained with the vanillin-HCl reaction. Either no effect is obtained or a secondary color is developed which obscures the true tryptophane color.

### *III. Effect of Sodium and Chloride Ions on Precipitation of Tryptophane by Mercuric Sulfate.*

The retarding effect of 0.05 per cent pepsin in 0.2 per cent HCl on the precipitation of tryptophane by mercuric sulfate in 9 per cent sulfuric acid was observed in my studies, as given in the following paper.

Onslow (4) claims "if pure caseinogen is hydrolyzed with 10 per cent NaOH or Ba(OH)<sub>2</sub> containing sodium salt no trace of mercury precipitate is obtained." He states that samples of commercial caseinogen were found to behave very differently from a pure product in that the apparent yield of tryptophane was only about 40 per cent of that from pure casein. He considers this to be due to the fact that the commercial product contains a large amount of sodium. The amount of sodium from 0.0005 gm. of pepsin (1 cc. of 0.05 per cent solution was used) did not promise to be a serious factor. From previous work and from studies carried out by Austin<sup>1</sup> it was known that HCl dissolves mercury-tryptophane precipitate. The exact concentration of HCl which will prevent precipitation of tryptophane by mercuric sulfate was not known.

Accordingly known amounts of pure tryptophane (0.2 mg.) were added to solutions containing varying but known concen-

<sup>1</sup> W. C. Austin, unpublished experiments from this laboratory.

trations of sodium chloride, hydrochloric acid, and sodium sulfate respectively. Upon these solutions the usual precipitation by mercuric sulfate in sulfuric acid and the subsequent estimation of the tryptophane in the precipitate were attempted. It was found that a 0.15 per cent concentration of chloride ion does not interfere with the quantitative recovery of tryptophane, that a 0.3 per cent concentration interferes seriously, and a 0.77 per cent or higher concentration practically inhibits the precipitation completely. This was true for both HCl and NaCl. Sodium sulfate on the other hand, even in 2 per cent concentrations (calculated to sodium) had no effect whatever. This shows that sodium is not a factor in retarding the precipitation of tryptophane by mercuric sulfate in a pepsin-HCl medium. Chloride is a great factor in concentrations of 0.3 per cent or more but in the pepsin-hydrochloric acid mixture employed in my studies the chloride concentration (0.02 per cent) is not great enough to cause the retardation. Furthermore, when the HCl is eliminated by use of an equivalent acidity of oxalic or acetic acid for peptic action the same inhibiting effect is noted. Therefore, some other factor enters in which as yet is unknown to the writer

#### SUMMARY.

1. A slight modification in the procedure of the vanillin-HCl reaction is made to save time and material.

2. The vanillin-HCl reaction applied directly to sixteen highly purified proteins gave very unsatisfactory results, the same as were shown in the previous publication.

3. When the same proteins were first hydrolyzed by trypsin and the indirect vanillin-HCl reaction applied (which means precipitation of tryptophane by mercuric sulfate under definite conditions) good results were obtained.

4. The filtrate from the mercury-tryptophane precipitate was worked up and shown definitely not to contain any tryptophane.

5. Peptide tryptophane if present in the mercury-tryptophane precipitate is shown to react the same as free tryptophane with the vanillin-HCl reaction.

6. Proline or proline-containing proteins, such as gelatin, in the concentrations used by Komm have no effect on the vanillin-

HCl color reaction but in higher concentrations a secondary color forms which interferes with the true tryptophane color.

7. Chloride ion concentrations of 0.3 per cent or higher interfere with the quantitative precipitation of tryptophane by mercuric sulfate, while sodium ion concentrations up to 2 per cent have no effect.

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## THE RATE WITH WHICH TRYPTOPHANE IS LIBERATED FROM PROTEINS BY ENZYMES.

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Hunter (1) carried out studies on the rate of liberation of amino acids from proteins by trypsin and states that "hydrolysis under the influence of a powerful trypsin preparation proceeds at first with a velocity which in some instances might almost be 'called' explosive." In the case of casein, Hunter and Smith (2) in one experiment found that nearly 45 per cent of the peptide groups was split in 1 hour. Nevertheless, it has long been believed that there is a certain sequence in the appearance of individual amino acids or groups of acids. Thus tyrosine, cystine, and tryptophane are thought to be among the first to appear and to be liberated completely in a relatively short period of time, while the hexone bases have usually been thought of as appearing rather late in tryptic digestion and it is considered that some, like proline, cannot be freed at all by trypsin alone (3). Hunter and Dauphinee (4) used the arginase method for arginine determination and were greatly surprised to find that arginine appears as soon as, if not earlier than, any other amino acid. They show that more than half of the arginine from casein and gelatin is liberated at the end of the first 3 hours and that an equilibrium is reached within 3 days. It was thought worth while to note the rate with which tryptophane is liberated from proteins by trypsin.

2 gm. of each of the following proteins were weighed into 250 cc. Erlennmeyer flasks. After the addition of 1 gm. of U.S.P. pancreatin, 100 cc. of 0.4 per cent  $\text{Na}_2\text{CO}_3$ , and toluene the mixtures were incubated at  $37^\circ$ . A blank containing no protein but pancreatin, etc., was treated in the same way. An aliquot or 5 cc. portion was taken immediately and at different intervals of time. 3 or 4

drops of 50 per cent  $\text{H}_2\text{SO}_4$  were added and filtered. A portion of the clear filtrate was used for tryptophane determination by the indirect vanillin-HCl (5) reaction in the usual manner. The results after correction for the tryptophane in the pancreatin are given in Table I.

Table I shows that one-third of the total potential tryptophane in Witte's peptone was free before incubation with trypsin and at the end of 1 hour's incubation an additional one-third was liberated. In the 1st hour three-fourths of the total available tryptophane is liberated in the case of casein, a little less than one-half

TABLE I.

Time.	Per cent tryptophane.			
	Casein.	Edestin.	Witte's peptone.	Squash seed globulin.
0	0	0	1.01	0
30 min.	0.58	0.45	1.16	0.34
hrs.				
1	0.92	0.60	1.91	0.73
5	1.01	0.73		0.83
24	1.09	0.80	3.05	1.01
48	1.15	0.89	3.05	1.54
72	1.20	0.91		1.75
96	1.23	1.12		
120	1.23	1.30		1.87
144	1.20	1.29	3.07	1.89
168		1.28		1.87

is liberated from edestin, and two-fifths from squash seed globulin. Equilibrium is established in Witte's peptone in 24 hours, in the case of casein in 72 hours to 96 hours, in edestin and squash seed globulin in 120 hours. It has been observed that the latter two proteins are not as soluble in 0.4 per cent  $\text{Na}_2\text{CO}_3$  as are the former. It was thought the more gradual liberation of tryptophane may be due to a difference in solubility, rather than a difference in enzyme action. Even after 48 hours of incubation with trypsin, on acidification with sulfuric acid a definite precipitate is formed in the edestin tube, squash seed tube, but none in the peptone tube, and just a trace in the casein tube.

Frankel (6) found that most proteins may be digested into solu-

tion with pepsin-HCl in several hours so that the surface factor may be eliminated. He also states that proteins after having been acted upon by pepsin are more readily digested by trypsin or erepsin. The fact that tryptophane from Witte's peptone (probably a peptic digestion product) is liberated and reaches an equilibrium in 24 hours or less suggested it as worth while to note the effect of the action of pepsin, trypsin, and erepsin on the liberation of tryptophane from such proteins as casein, edestin, and squash seed globulin. Thus the three proteins mentioned were subjected to the action of the three enzymes in the order named, with concentrations and procedure as given by Frankel (6) except instead of aliquot portions being taken at short intervals of time for a determination during peptic digestion, a portion was taken practically at the end of the pepsin period. Thus after 96 hours incubation with pepsin-HCl a portion was removed and analyzed for amino nitrogen by the micro method of Van Slyke (7) and for tryptophane by the indirect vanillin-HCl method. The remainder of the digest was subjected to tryptic action<sup>1</sup> and portions taken at intervals as in the previous experiment for tryptophane determination, *etc.*

In the analysis of the peptic hydrolysate for tryptophane a small amount or none was expected, consequently a known amount of tryptophane was added to each tube as well as to the blank and the standard (plan followed throughout the work where a trace was expected). A peculiar thing was observed. In all tubes excepting the standard the mercury-tryptophane failed to precipitate in 2 hours.<sup>2</sup> The tubes were allowed to stand for 24 hours. Then a rather heavy precipitate formed, the lower layer being yellow, characteristic of mercury-tryptophane, covered by an upper white layer. The tubes were centrifuged, *etc.*, the reaction completed, and colorimetric readings made after 24 hours. The tubes containing the hydrolysate showed a different tint of color and accurate readings could not be made, indicating that a substance other than tryptophane was carried down by the mercury precipitation; *i.e.*, some undigested protein evidently was carried down. The control tube which contained no proteins

<sup>1</sup> The solutions were preserved with toluene instead of tricresol.

<sup>2</sup> 2 hours is the period of time found to be sufficient for the quantitative precipitation under conditions as given in the vanillin-HCl method (5).

excepting the enzyme pepsin, precipitated completely after 36 hours and the reading was slightly higher than that of the standard.<sup>3</sup> The analysis was repeated without the addition of any pure tryptophane to the tubes containing hydrolysates to note whether any precipitate would form to give a test for tryptophane. After 24 hours standing a slight white precipitate formed which, when

TABLE II.

Enzyme.	Hrs.	Casein.		Edestin.		Squash seed.	
		Total NH <sub>2</sub> -N 9.93 per cent.		Total NH <sub>2</sub> -N 11.32 per cent.		Total NH <sub>2</sub> -N 11.29 per cent.	
		Trypto- phane.	NH <sub>2</sub> -N	Trypto- phane.	NH <sub>2</sub> -N	Trypto- phane.	NH <sub>2</sub> -N
		per cent	per cent	per cent	per cent	per cent	per cent
Pepsin.	96	Trace (?)	16	Trace (?)	18	Trace (?)	17
Trypsin.	$\frac{1}{2}$	0.60	22	0.48	25	0.37	31
	1	1.00		0.63		0.75	
	5	1.05		0.78		0.87	
	24	1.10		0.85		1.05	
	48	1.15		0.95		1.54	
Trypsin.	72	1.20		1.10		1.80	
	96	1.22	63	1.28		1.83	64
	120	1.20	65	1.30	64	1.87	65
	144			1.31	65	1.88	
	168			1.30	67	1.85	69
Erepsin.	24	1.19	81	1.29	79	1.87	83
	48	1.17	84			1.86	86
	72	1.00	87	1.25	85		
Hydrolysis completed by acid.		Slight tint.	99.3		100.92		100.8

the vanillin reaction was applied, showed a trace of tryptophane but not enough to estimate. The trace undoubtedly is due to the

<sup>3</sup> Calculations based on the reading obtained show pepsin to contain 1.06 per cent tryptophane. Hugounenq and Morel (8) analyzed pepsin for the different amino acids and reported "negative" for tryptophane. A 1 per cent pepsin in 0.2 per cent HCl solution was prepared and a direct vanillin reaction for tryptophane made. Although a slight reddish hue was present, yet averaging several determinations made on different concentrations showed pepsin to contain 0.98 per cent tryptophane.

pepsin since the control showed a similar trace. Thus, pepsin did not change these proteins to a form in which free tryptophane can be precipitated.

The digestions were continued with the other enzymes to note whether any differences will be observed in the rate and amount of tryptophane liberation from the proteins first acted on by pepsin.<sup>4</sup>

It is apparent from the results in Table II that subjecting a protein first to pepsin and then to trypsin does not hasten or increase the liberation of tryptophane; also, that only the enzyme or enzymes in U.S.P. pancreatin liberate tryptophane from the proteins studied. Although the other two enzymes definitely liberate amino nitrogen they do not liberate tryptophane. It was planned to continue the experiment until the liberation of amino nitrogen came to an equilibrium but since the per cent of free tryptophane began to diminish, the experiment was concluded by completing the hydrolysis with acid. Portions of each of the hydrolysates and of the blank were made acid (20 per cent by volume with sulfuric acid) and heated on an electric plate for 24 hours. The percentages of total amino nitrogen obtained, as shown in Table II, after acid hydrolysis, indicate no loss of amino nitrogen. The results on amino nitrogen in Table II confirm the findings of Frankel; i.e., that in a relatively short time extensive hydrolysis takes place, that peptic activity liberates close to 20 per cent of the potential amino nitrogen, trypsin about 60 to 70 per cent, and erepsin about 20 per cent.

#### SUMMARY.

1. The indirect vanillin-HCl reaction has been applied in studying the rate with which tryptophane is liberated from proteins by enzymes.

2. Pepsin action does not liberate tryptophane from the proteins studied. Erepsin, if used after the action of trypsin, does not liberate additional tryptophane from casein, edestin, and squash seed globulin.

<sup>4</sup> In this experiment, the precipitation of tryptophane by mercuric sulfate was allowed a longer period of time (24 to 36 hours) because of the retarding effect of pepsin and HCl. In the paper preceding it is shown that the chloride concentration involved here is not sufficient to interfere with the quantitative precipitation of tryptophane.

3. Tryptophane is liberated more readily from casein than from edestin and squash seed globulin by trypsin (U.S.P. pancreatin), and equilibrium is reached sooner than in the latter two proteins.

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## ANTIRICKETIC SUBSTANCES.

### IX. QUANTITATIVE BIOPHYSICAL STUDIES ON THE ACTIVATION OF ERGOSTEROL.\*

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Characteristic of ergosterol are the absorption bands which it exhibits in the ultra-violet spectrum. Pohl (1) and Morton, Heilbron, and Kamm (2) have shown that when ergosterol is irradiated with ultra-violet light the three bands having maxima at 293.5, 282, and 270  $m\mu$  disappear, while a new band at 247  $m\mu$  becomes evident. On the basis of physical studies, without biological confirmation, Pohl assumed that the 247  $m\mu$  band represents vitamin D. Morton, Heilbron, and Kamm (2, 3) arrived at the same conclusion, stating that an incidental feeding experiment supported their hypothesis. Rosenheim and Webster (4) have called attention to the danger of misinterpreting the spectroscopic data in the absence of sufficient biological control.

We have investigated the activation of ergosterol by a series of parallel spectroscopic and biologic tests. The ergosterol was a product of exceptional purity,  $[\alpha]_D^{20} = -132^\circ$  in  $\text{CHCl}_3$  (Bills and Honeywell (5)). It was dissolved, exactly 1 gm. to a liter, in optically pure 95 per cent alcohol. When not in use, this stock solution was stored in the dark at low temperature. Under these conditions there occurred no deterioration detectable with the spectroscope.

The solution was activated in a homogeneous quartz absorption cell, 2 cm. deep, completely filled (16 cc.), and tightly corked. The cell was placed in contact with the window of a Kromayer

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lamp, being thus separated from the burner by less than 2 cm. of water. It should be noted that the emission spectrum of water-cooled mercury lamps is not only intrinsically intense, but relatively more intense in the "destructive" short wave-length radiations than is that of the air-cooled types. In following the changes taking place after different time intervals we did not withdraw successive samples from the cell and thereby leave more or less air above the residual solution; instead, we used a fresh cellful for each period of exposure. Thus the solution was protected to a large extent from radiant heat, air, and ozone. The lamp was operated at nearly uniform voltage and temperature, so that the light must have been reasonably constant.

The absorption curves (Fig. 1) were made with a Hilger quartz spectrograph, sector photometer, and tungsten steel spark. The activation curve (Fig. 2) was plotted from tests made with rats according to the method previously described (6). About twenty rats were used to establish each point on this curve, after preliminary tests had given the approximate position. We are confident that the quantitative method which we have practiced on 7000 animals gives to the activation curve a degree of accuracy closely approaching that of the absorption curves. We used a portion of the irradiated alcoholic ergosterol solution for photographing the absorption spectra, and immediately thereafter we diluted the remainder with peroxide-free ether 1:10. This diluted solution was then further diluted with fresh cottonseed oil 1:20 or 1:10, to give a stable solution of convenient strength for incorporating with Diet 3143 (7).

Preliminary trials indicated that in order to obtain an exact portrayal of the course of activation, it was necessary to irradiate the solution for the following periods:  $7\frac{1}{2}$ , 15,  $22\frac{1}{2}$ , 30, 45 minutes, 1, 2, 3, 4, 5,  $7\frac{1}{2}$ , 10, and 15 hours. Figs. 1 and 2 reveal the order of change.

*Non-Irradiated Stock Solution.*—The high molecular extinction coefficient, 11,200, indicates the unusual purity of the ergosterol. The absorption bands with maxima at 270 and 282  $m\mu$  are prominent; the band at 293.5  $m\mu$  is evident, although less well defined. The fourth ergosterol band, 260  $m\mu$ , which MacNair (6) recently detected in continuous light source photographs, is too feeble to be recognized with the spark. No antiricketic activity is evident.



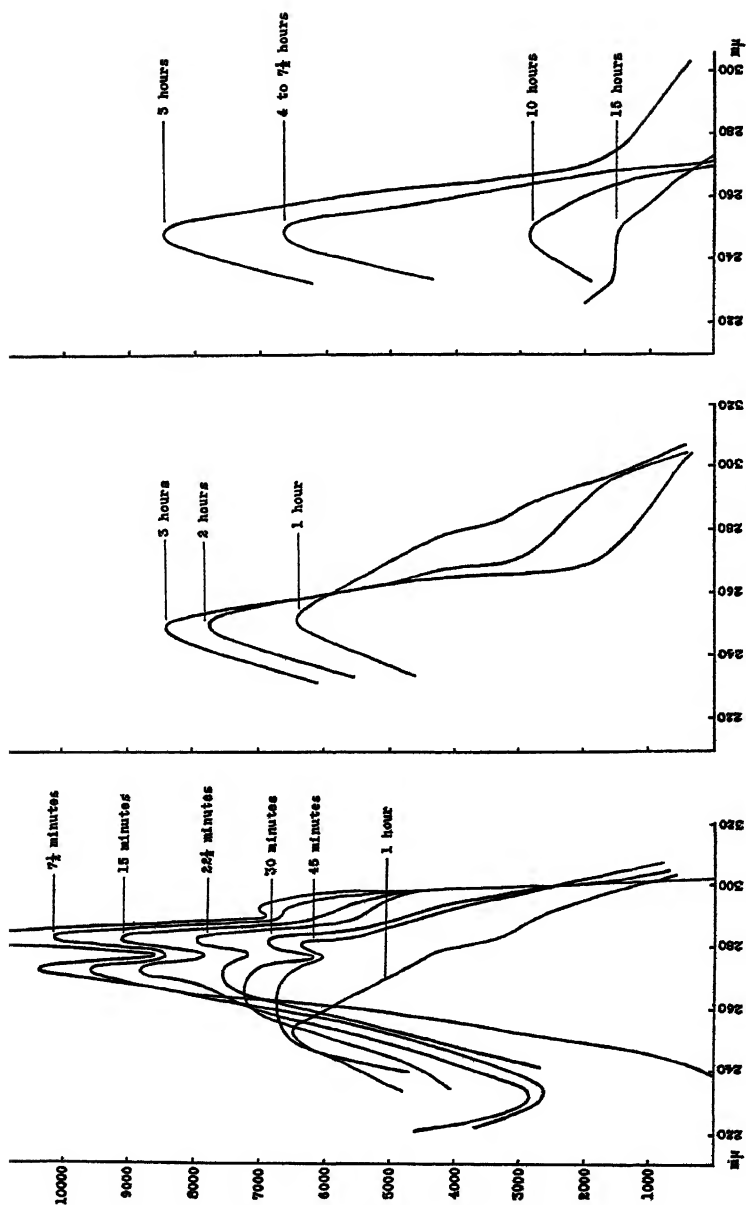


FIG. 1. Effect of irradiation on the ultra-violet absorption of ergosterol.

*Irradiated 7½ Minutes.*—The absorption curve closely resembles that of the non-irradiated solution, the maxima being only a little lower. There is no evidence of any new absorption band, yet the animal tests reveal the development of enormous antiricketic potency.

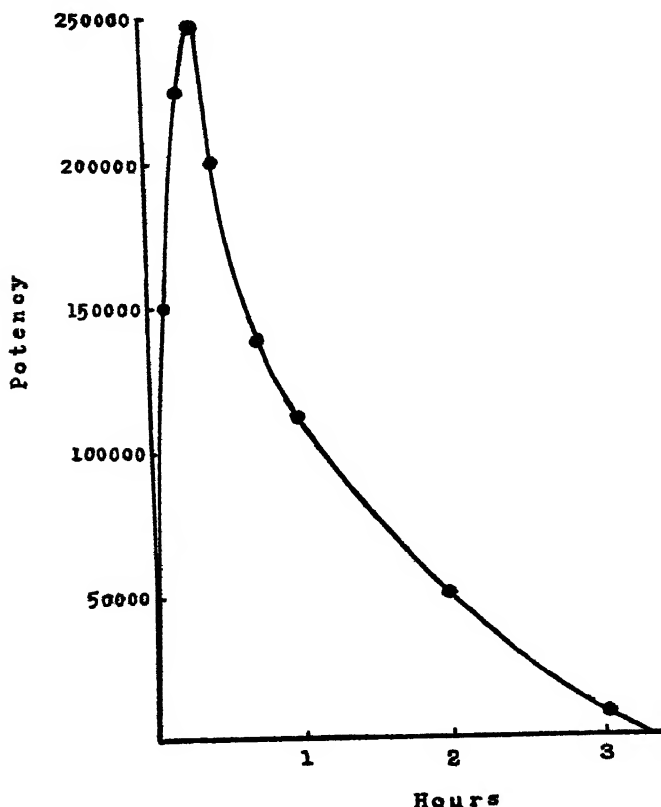


FIG. 2. Effect of irradiation on the antiricketic potency of ergosterol.

The ergosterol has become 150,000 times as potent as average cod liver oil.

*Irradiated 15 Minutes.*—As yet there is only slight change in the absorption curve, and no evidence of a new band. The activation has nearly reached its maximum, the potency being 225,000  $\times$ .

*Irradiated 22½ Minutes.*—The band at  $270\text{ m}\mu$  has broadened slightly; otherwise there is little change in the absorption curve. The activation curve has reached its highest point, the potency being  $250,000 \times$ .

*Irradiated 30 Minutes.*—The maximum at  $293.5\text{ m}\mu$  has disappeared, that at  $282\text{ m}\mu$  is still conspicuous, and that at  $270\text{ m}\mu$  shows a distortion that indicates the incipient development of a new region of absorption in the shorter wave-lengths. The potency has declined to  $200,000 \times$ .

*Irradiated 45 Minutes.*—The maximum at  $282\text{ m}\mu$  has faded considerably, and that at  $270\text{ m}\mu$  has shifted more definitely to the shorter wave-length region. The antiricketic potency continues to decline.

*Irradiated 1 Hour.*—The maximum at  $282\text{ m}\mu$  has almost disappeared, that at  $270\text{ m}\mu$  is barely evident, and the new maximum is becoming distinct at about  $252\text{ m}\mu$ . The potency continues to decline.

*Irradiated 2 Hours.*—All the absorption maxima of ergosterol have vanished. The new band, however, has become more intense; its maximum now lies at about  $250\text{ m}\mu$ , and the extinction coefficient has risen to 7800. The antiricketic potency has declined to  $50,000 \times$ .

*Irradiated 3 Hours.*—Coincident with the development of the new band in its greatest intensity, the antiricketic potency has almost vanished. The new extinction coefficient is 8400, and the absorption maximum lies at  $248\text{ m}\mu$ .

*Irradiated 4, 5, 7½, 10, and 15 Hours.*—Between 3 and 4 hours the absorption band at  $248\text{ m}\mu$  fades slightly; during 4 to  $7\frac{1}{2}$  hours no further change is observed; after  $7\frac{1}{2}$  hours the fading resumes. Antiricketic potency never reappears.

It is thus evident that the photochemical reaction product which exhibits the absorption band at  $248\text{ m}\mu$  is not vitamin D; for its appearance coincides not with the development, but with the destruction of antiricketic potency. It must be either a by-product of the activation of ergosterol, or a degradation product of the vitamin.

The wave-lengths which vitamin D itself absorbs, and by which it is destroyed, apparently lie within the same spectral region as the wave-lengths which activate ergosterol. This explains why

Heilbron, Kamm, and Morton (3) were unable to separate by filters the activating from the destroying rays, and thus guide the reaction to a more effective activation. Clearly, the spectroscope cannot replace the biological method in studying the formation of vitamin D.

The activation curve (Fig. 2) merits special attention. Considered in connection with the absorption curves, it shows that antiricketic potency is developed rapidly, reaching its maximum when about 73 per cent of the ergosterol is still present. The decline in potency commences sharply at the point where, on account of a depleted reserve of ergosterol and a consequent decline in the rate of formation of vitamin, the decomposition overtakes the formation. Obviously, the activation curve, as well as the absorption curves, is the resultant of numerous influences not as yet understood. The activation curve merely shows the potency of the reaction product at any given time; it reveals little as to the formation distinct from other changes.

In another series of experiments we plotted the absorption and activation curves for an ergosterol solution which was irradiated according to the procedure of Morton, Heilbron, and Kamm (2). The difference in technique consisted in the use of but one cell of solution, from which samples were withdrawn from time to time, leaving more and more air in contact with the residual liquid. When allowances for differences in the intensity of the light source and purity of the ergosterol were made, the absorption curves were strikingly similar to those obtained by the English investigators. Compared with the curves above described, this second series differed in that the band at  $248\text{ m}\mu$  developed and faded much more rapidly, and never exhibited as high an extinction coefficient. The activation curve was, however, very similar to the one already shown in Fig. 2. A simple calculation reveals that the amount of oxygen in the air contained in a partly filled cell is sufficient for considerable oxidation of a 1:1000 ergosterol solution. It seems obvious that oxidation is involved in the destruction of the  $248\text{ m}\mu$  band, but not in the formation of vitamin D.

Whatever be the nature of the substance exhibiting the absorption band at  $248\text{ m}\mu$ , it is clearly related to ergosterol and vitamin D. One might suppose it to be a primary decomposition product or isomer. Indeed, we have discovered that the isoergosterol

of Reindel, Walter, and Rauch (8) exhibits just such a band. The absorption of isoergosterol extends from about 220 to 270  $m\mu$ , reaching a maximum at 248  $m\mu$ , and exhibiting a molecular extinction coefficient of 16,300. Upon prolonged irradiation this band fades much the same as does the band characteristic of the unknown substance. Nevertheless we are not at all certain that isoergosterol and this substance are actually identical. At present it is sufficient to emphasize that along with the formation of vitamin D by light, there is formed a substance which must have a molecular configuration not unlike that of isoergosterol.

#### SUMMARY.

1. A series of parallel spectroscopic and biologic measurements was made on an irradiated alcoholic solution of exceptionally pure ergosterol. It was found that the photochemical reaction product which exhibits an absorption maximum at 248  $m\mu$  is not vitamin D; for the appearance of this band coincides not with the development, but with the destruction of antiricketic activity.

2. The rise and decline of antiricketic potency has been accurately depicted by means of a graph plotted from tests on a large number of animals.

3. Although oxidation plays no part in the formation of vitamin D, it appears to be a factor in the destruction of the substance which exhibits the absorption band at 248  $m\mu$ .

4. It is suggested that the absorption band at 248  $m\mu$  is due to a substance having a molecular configuration similar to that of isoergosterol.

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## THE MANGANESE-COPPER-IRON COMPLEX AS A FACTOR IN HEMOGLOBIN BUILDING.\*

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In an earlier paper Titus and Cave (1) presented data showing that manganese is effective in hemoglobin building in the nutritional type of anemia when the rabbit was used as the experimental animal. Hart and coworkers (2) presented data to the effect that copper is a factor in the building of hemoglobin when the rat was used as the experimental animal. Previous to this report these same investigators (3) showed that high purity iron salts when fed at 0.5 mg. levels to rats were ineffective in correcting a nutritional type of anemia brought about by feeding a diet of cow's whole milk.

Our attention was directed toward a study of manganese when rabbits, which had been fed for some time a corrective diet containing  $\text{Fe}_2\text{O}_3$  of "analyzed" purity, continued to grow and failed to become anemic as we had expected. About this time Professor E. B. Hart of the University of Wisconsin very kindly sent one of us a small amount of  $\text{Fe}_2\text{O}_3$  which he had found to be ineffective in preventing this nutritional type of anemia. Feeding this oxide of iron to our rabbits confirmed his experience in this respect, as our rabbits rapidly became anemic. We began to make comparisons between these two samples of  $\text{Fe}_2\text{O}_3$  and found, among other things, that the "analyzed" sample of  $\text{Fe}_2\text{O}_3$  contained about 4 times as much manganese as the sample which we received from Professor Hart. We also investigated the lettuce ash and found it to be quite high in manganese. The bluish green color which appeared

\* Contribution No. 145, Department of Chemistry, and No. 63, Department of Dairy Husbandry.

on strong ignition we attributed not to copper, but to a manganese compound, possibly combined in some way with some of the potash which is so abundant in our samples of lettuce.

In this paper we wish to report the beneficial results obtained when (a) copper was added to a milk-iron diet, (b) manganese was added to a milk-iron diet, (c) manganese and copper were added to a milk-iron diet. In this particular work the rat was used as the experimental animal.

TABLE I.  
*Diet of Liquid Whole Milk + 0.5 Mg. of Fe.*

Rat No.	Date.....	Sept. 11.	Sept. 17.	Sept. 24.	Oct. 1.	Oct. 8.	Oct. 15.
121	Hb, per cent.*	5.02	5.30	6.94	7.62	7.48	7.88
	Weight, gm.	76	90	96	102	110	104
122	Hb, per cent.	5.44	5.98	6.12	5.02	4.62	4.62
	Weight, gm.	85	91	92	82	97	100
123	Hb, per cent.	5.16	4.90	5.44	5.58	5.44	5.72
	Weight, gm.	147	153	151	146	132	117
124	Hb, per cent.	5.16	4.36	Dead.			
	Weight, gm.	114	108				
125	Hb, per cent.	6.12	3.00	Dead.			
	Weight, gm.	113	104				
126	Hb, per cent.	9.04	6.26	8.42	8.98	9.24	10.34
	Weight, gm.	133	140	140	127	144	144
127	Hb, per cent.	5.98	4.62	7.88	8.56	5.98	5.48
	Weight, gm.	110	113	112	108	119	119
128	Hb, per cent.	4.76	3.54	Dead.			
	Weight, gm.	90	80				
129	Hb, per cent.	9.24	6.80	6.94	7.34	7.48	5.58
	Weight, gm.	111	114	105	89	91	81
130	Hb, per cent.	7.34	6.26	Dead.			
	Weight, gm.	92	94				

\* Hb, per cent = gm. of hemoglobin per 100 cc. of blood.

#### EXPERIMENTAL.

Rats at about 4 weeks of age were taken from their mothers and placed on a liquid whole milk diet until the hemoglobin content of their blood was sufficiently reduced. As suggested by Waddell and coworkers (4) the time required to reduce the hemoglobin to below 5 gm. per 100 cc. of blood depends very largely upon the ration fed to the mother during her period of gestation. The time



required to produce this anemic condition varied from 2 to 6 weeks. The animals were ear-marked and placed in individual wire cages with screen bottoms. The food dishes used were ordinary porcelain mortars. Fresh whole cow's milk was fed *ad libitum*. The corrective supplement was added to a scant morning feed in order to insure consumption of the entire mineral supplement. Later in the day, when the food containing the supplement had been consumed, a liberal feeding of milk was given each animal.

TABLE II.  
*Diet of Liquid Whole Milk + 0.5 Mg. of Fe + 0.05 Mg. of Cu.*

Rat No.	Date.....	Sept. 8.	Sept. 14.	Sept. 17.	Sept. 24.	Oct. 1.	Oct. 8.	Oct. 15.
111	Hb, per cent.*	3.54	8.70	11.82	13.06	15.64	15.78	14.96
	Weight, gm.	93	116	118	145	133	160	159
112	Hb, per cent.	2.44	8.98	10.34	15.51	16.04	15.78	14.42
	Weight, gm.	104	127	128	135	135	165	172
113	Hb, per cent.	2.30	8.70	7.34	9.24	12.78	13.06	14.28
	Weight, gm.	67	92	104	115	118	137	134
114	Hb, per cent.	5.30	10.34	11.56	14.56	15.22	14.96	12.78
	Weight, gm.	92	114	120	141	138	173	183
115	Hb, per cent.	4.36	6.12	8.16	9.52	14.28	13.06	13.73
	Weight, gm.	70	87	104	135	130	168	178
116	Hb, per cent.	5.30	9.92	12.24	10.88	16.72	14.96	15.22
	Weight, gm.	93	111	119	130	132	143	137
117	Hb, per cent.	5.16	7.34	9.24	11.42	15.10	14.42	14.42
	Weight, gm.	70	86	99	117	128	139	147
118	Hb, per cent.	2.30	10.34	13.06	14.42	13.86	13.86	14.42
	Weight, gm.	109	130	135	163	175	188	187
119	Hb, per cent.	5.72	10.20	Dead.				
	Weight, gm.	82	90					
120	Hb, per cent.	4.08	5.72	Dead.				
	Weight, gm.	105	101					

\* Hb, per cent = gm. of hemoglobin per 100 cc. of blood.

The animals were weighed and hemoglobin determinations made each week. In obtaining blood samples, instead of snipping the tail, as is the most general practice, we found that sufficient blood could be obtained for our purposes by pricking the caudal vein with a bleeding knife or sharp scalpel. This vein is easily observed by placing the rat on a table and slightly rotating the tail until the vein is uppermost. A slight prick a few cm. from the end of the

tail is generally sufficient to give a good drop of blood. We have found this method much superior to that of snipping the tail and much better for the animal.

Hemoglobin determinations were made with a Fleischl-Miescher hemoglobinometer. In Table I are recorded hemoglobin and growth data for a group of rats on a milk-iron diet. This iron was prepared from Mallinckrodt's iron wire such as is used for

TABLE III.  
*Diet of Liquid Whole Milk + 0.5 Mg. of Fe + 0.1 Mg. of Mn.*

Rat No.	Date, .....	Aug. 27.	Sept. 3.	Sept. 10.	Sept. 17.	Sept. 24.	Oct. 1.	Oct. 8.	Oct. 15.
101	Hb, per cent.*	5.49	9.78	12.78		15.52	14.96	15.84	16.04
	Weight, gm.	87	100	112	120	129	125	133	133
102	Hb, per cent.	4.50	7.74	10.88	9.78	14.14	15.50	14.68	14.68
	Weight, gm.	93	109	123	133	150	126	152	154
103	Hb, per cent.	4.08	4.08	4.08	4.90	8.84	10.88	12.10	13.19
	Weight, gm.	86	77	95	104	111	103	124	150
104	Hb, per cent.	4.88	5.44	6.94	6.52	8.98	10.34	10.06	11.14
	Weight, gm.	77	103	110	126	135	124	140	139
105	Hb, per cent.	4.08	4.76	4.08	6.26	6.80	10.06	9.52	9.92
	Weight, gm.	75	75	80	98	100	89	116	125
106	Hb, per cent.	2.85	5.16	9.38	9.52	14.42	16.85	15.34	14.55
	Weight, gm.	64	84	100	120	131	120	138	150
107	Hb, per cent.	3.45	3.54	4.48	5.58	8.42	10.20	7.75	9.24
	Weight, gm.	71	77	75	90	92	90	100	112
108	Hb, per cent.	2.64	5.44	9.92	11.56	14.42	16.16	14.55	14.55
	Weight, gm.	85	100	119	132	150	123	158	167
109	Hb, per cent.	3.66	3.54	4.48	5.44	7.08	8.56	7.34	8.16
	Weight, gm.	65	73	75	92	89	83	109	114
110	Hb, per cent.	5.70	9.78	13.46	12.24	15.52	17.12	13.32	13.46
	Weight, gm.	81	92	98	106	116	99	126	136

\* Hb, per cent = gm. of hemoglobin per 100 cc. of blood.

standardizing. The wire was dissolved in a minimum amount of Mallinckrodt's reagent quality hydrochloric acid, then treated with hydrogen sulfide under pressure for 12 hours in order to precipitate any copper which might be present.

Rats having a fairly high hemoglobin in their blood at the beginning of the experiment seemed to be able to utilize the iron to some extent, while the animals with the low hemoglobin to begin with were unable to utilize the iron when fed without copper or manganese supplement.

In Table II are recorded the hemoglobin and growth data for a group of rats on a milk-iron diet supplemented with 0.05 mg. of copper per rat per day. This is the optimum amount of copper as reported by Hart (2). The copper solution was fed in the form of the sulfate, prepared from the purest electrolytic copper obtainable, dissolved in Mallinckrodt's reagent quality sulfuric acid. Good growth and increased hemoglobin are shown by all the ani-

TABLE IV.

*Diet of Liquid Whole Milk + 0.5 Mg. of Fe + 0.05 Mg. of Cu + 0.1 Mg. of Mn.*

Rat No.	Date.....	July 31.	Aug. 7.	Aug. 14.	Aug. 21.	Aug. 28.	Sept. 3.	Sept. 10.	Sept. 16.
51	Hb, per cent.*	2.72	12.24	14.28	13.26	14.46	14.28	14.28	15.64
	Weight, gm.	55	77	94	102	107	105	123	127
52	Hb, per cent.		6.66	14.96	13.26	14.28	14.68	14.28	13.86
	Weight, gm.		58	85	87	93	99	116	132
53	Hb, per cent.	6.26	10.74	13.32	13.86	13.47	14.96	14.00	13.92
	Weight, gm.	59	80	105	106	111	121	130	130
54	Hb, per cent.	6.94	13.05	14.42	14.28	13.86	13.32	13.72	14.96
	Weight, gm.	54	75	99	101	112	125	134	145
55	Hb, per cent.	6.80	6.26	15.78	13.65	14.46	14.96	14.28	14.28
	Weight, gm.	62	70	95	96	101	103	119	130
56	Hb, per cent.		6.12	15.04	12.24	14.07	15.24	14.42	14.68
	Weight, gm.		50	80	94	104	111	129	144
57	Hb, per cent.	6.12	12.92	15.64	14.07	13.65	14.14	14.00	14.42
	Weight, gm.	57	80	97	98	106	118	123	125
58	Hb, per cent.		6.38	12.50	12.84	13.86	14.82	14.42	14.96
	Weight, gm.		59	90	98	110	112	127	137
59	Hb, per cent.		5.16	15.78	12.63	14.28	13.5	12.92	14.42
	Weight, gm.		63	90	93	99	103	115	127
60	Hb, per cent.	5.58	10.88	11.82	13.26	14.07	16.32	14.14	14.96
	Weight, gm.	50	72	84	90	98	102	105	112

\* Hb, per cent = gm. of hemoglobin per 100 cc. of blood.

mals in this lot. This is in accord with the findings of Hart and co-workers at Wisconsin (2).

In Table III are recorded weight and hemoglobin data for animals on a milk-iron diet supplemented with 0.1 mg. of manganese per animal per day. The manganese used was in the form of the chloride and prepared by treating manganous carbonate, Baker and Adamson quality, with the minimum amount of Mallinck-

rod's reagent quality hydrochloric acid. This solution was then treated with hydrogen sulfide under pressure for 12 hours in order to remove the copper that might be present. The manganese carbonate was found to contain about 0.002 per cent copper. Even if the copper had not been removed, the amount fed with the manganese supplement would probably have been too small to have given any beneficial results.

For the most part the rats in this lot showed a good growth and increase in their hemoglobin. A few, however, failed to show any great response to the supplement.

Data are presented in Table IV for a group of animals on a milk-iron diet supplemented with both copper and manganese.

These animals showed the most rapid response in hemoglobin of any of the groups and gave each week a more constant hemoglobin reading. This would seem to be in line with the suggestions made by Whipple (5) relative to the potency of liver extracts in which he says "the evidence points to a group of active substances rather than to a single active principle."

#### SUMMARY.

1. Manganese added to a milk-iron diet seems to give almost, if not quite, as good results in the building of hemoglobin as does copper added in the same way.

2. Manganese and copper added to a milk-iron diet appear to produce a quicker response from the standpoint of hemoglobin building than does either copper or manganese when fed alone as a supplement.

3. Experimental data presented seem to indicate the existence of a group of substances, rather than a single substance, which is active in hemoglobin building.

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# THE RELATIONSHIP OF LACTIC ACID TO THE OPTICAL ACTIVITY OF NORMAL AND DIABETIC BLOOD BEFORE AND AFTER GLYCOLYSIS.

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In previous work by one of us upon the reducing and rotatory properties of plasma filtrates (1, 2), the literature upon this subject has been briefly reviewed and the optically active substances other than glucose have been discussed, although no attempt has been made to define their nature. Reference has been made also to the fact that in the course of the glycolytic action of whole blood the levorotatory substances or at least levorotation increases, whereas, if the blood sugar is removed by fermentation with yeast, no such increase of levorotation is recorded. It was also noted that levorotation was increased in the blood immediately after death, a phenomenon which was generally associated with low blood sugar values.

In continuing our studies upon these optically active substances it became evident that in many of the blood samples examined, the degree of levorotation appeared to be a function of glycolysis and was apparently dependent, more or less quantitatively, upon the original amounts of glucose present, or in other words levorotatory substances were being formed at the expense of the blood sugar, thus raising the question as to the relationship which these substances might bear to lactic acid.

We are aware that the exact processes which occur as a result of the glycolytic action of whole blood are somewhat obscure but it has long been known that in this reaction lactic acid is formed at the expense of glucose. The literature on this subject is quite

extensive and has been reviewed in the recent studies of Barron and Harrop (3). An important observation bearing on this process was made in 1912 by Karl von Noorden (4), who found that the lactic acid formed in the course of the glycolytic action of whole blood was *d*-lactic acid. He succeeded in isolating this substance from the blood in the form of its zinc salt which was levorotatory, as, he states, "are the salts of *d*-lactic acid." It has of course been appreciated that lactic acid is probably represented in the blood both normally and after glycolysis by one of its salts but the degree to which the levorotatory substances of the blood are represented by lactates has received relatively little attention. The suggestion has been made that lactic acid may be in part responsible for these levorotatory substances by Visscher (5) and others, but we are not aware of any work in which this relationship has been carefully studied.

#### *Methods.*

The methods employed in these experiments have been described in the previous communications (1, 2), and consequently only the essential features will be reviewed here.

Between 30 and 50 cc. of blood were required for the individual determinations and these were obtained under aseptic precautions from the arm vein in human beings and from the carotid artery in animals. In the early experiments small quantities of sodium citrate were added as an anticoagulant but in later ones the blood was defibrinated.

The filtrates were obtained by introducing 15 to 30 cc. of plasma into a collodion sac which was then placed in a partially evacuated flask. These sacs were prepared from a 7 per cent solution of du Pont's parlodion dissolved in a solution of 75 per cent by volume of ether, and 25 per cent by volume of 95 per cent alcohol. The standard adopted as to the suitability of the sacs was, that they should be impermeable to hemoglobin; but easily permeable to glucose. In making a sac the inside of a test-tube was coated with the collodion solution, drained, and then recoated; it was then drained again and allowed to dry until the collodion was quite firm. The tube was then filled with 70 per cent alcohol and allowed to stand for 10 minutes. Distilled water was then substituted for the alcohol for a period of 1 hour. At the end of this time the

sac was found to have contracted slightly from the tube wall and could be drawn out with relative ease. The sacs were first tested for leaks and impermeability to hemoglobin, by filling them with a solution of hemoglobin and subjecting them to a pressure difference of 200 mm. of mercury. The selected ones were kept in water to which a few crystals of thymol had been added and were often used repeatedly over a period of several days. The method of ultrafiltration was that described by Marshall and Vickers (6).

Polariscopic readings were made with a Reichert instrument, with a mercury vapor lamp (green light) as the light source. A special polariscope tube measuring 241.8 mm. in length and holding 10 cc. of solution was employed to make the determinations. This utilized the maximum length allowed by the instrument and at the same time did not require an excess of solution. The length of the tube was also gauged to facilitate the calculations for estimating the per cent of glucose, for by employing a wave-length of 5416 Å. the specific rotation of glucose is  $63.03^\circ$  and the percentage can be derived from the following simple formula.

$$\text{Per cent glucose} = \frac{\text{reading} \times 100}{62.03 \times 2.418} = \frac{\text{reading}}{1.5}$$

In determining the error shown by this method, readings were made on a series of standard glucose solutions of a concentration of approximately 0.100 to 0.150 gm. per 100 cc. The readings of the solutions were frequently alternated with readings of the zero point. The average error of the individual determination was about  $\pm 0.010$  gm. of glucose.

The reducing determinations were made by the method of Folin and Wu (7).

In determining lactic acid the procedure employed by Friedemann, Cotonio, and Shaffer was followed (8). 1 cc. portions of the filtrate were employed for each determination. The method was standardized by a series of determinations run on 20 cc. samples of 0.001 N solution of zinc lactate.<sup>1</sup> In a series of five such determinations run over a period of from 3 to 4 days 95 to 96 per cent of the theoretical yield of lactic acid was obtained.

<sup>1</sup> We are indebted to Dr. D. Wright Wilson of the Department of Physiological Chemistry for obtaining a supply of purified crystals of zinc lactate.

## EXPERIMENTAL.

We first engaged upon a series of studies on the relationship which the initial blood filtrate reducing values (corrected to designate the initial glucose content) would bear to the levorotatory values obtained after glycolysis. These were performed upon rabbit blood for it was found that the range of blood sugar levels existing in apparently normal rabbits is greater than that in human beings.

TABLE I.

*Relation of Initial Reducing Values to Postglycolytic Rotatory Values.*

The values are expressed in terms of gm. of glucose per 100 cc.

Before glycolysis.				After glycolysis.	
Plasma.	Ultrafiltrate.			Ultrafiltrate.	
Reducing values.	Reducing values.	Reducing values minus non-glycolyzable residue.	Rotatory values.	Reducing values or non-glycolyzable residue.	Rotatory values.
0.090	(0.100)*	(0.080)	0.109	0.020	-0.054
0.089	0.107	0.098		0.009	-0.089
0.112	(0.123)	(0.113)		0.010	-0.091
0.138	(0.152)	(0.146)		0.006	-0.083
0.165	(0.181)	(0.166)		0.015	-0.127
0.170	(0.187)	(0.171)		0.016	-0.154
0.173	(0.190)	(0.182)		0.008	-0.141
0.177	0.193	0.174	0.180	0.019	-0.154
0.183	0.195	0.181	0.182	0.014	-0.134

\* The values in parentheses are not direct observations but have been calculated from the plasma reducing values.

The experiments were carried out in the following manner: Fairly large rabbits were selected, either after fasting for 24 hours, or shortly after a meal. They were etherized, and samples of blood ranging from 60 to 80 cc. were obtained from the carotid artery through a bleeding cannula, sodium citrate being used as an anticoagulant. After an initial blood sugar determination the sample of blood was incubated at 37° and glycolysis was allowed to proceed for 16 to 20 hours. The plasma was then filtered through a collodion membrane and reducing and rotatory deter-



minations were made upon the resulting ultrafiltrates. In a few instances when the samples of blood were sufficiently large they were divided in half as soon as obtained. The plasma from the first sample was immediately filtered and initial reducing and rotatory determinations were obtained for comparison with the reducing and rotatory determinations obtained in the second sample after glycolysis. It was thought unnecessary, however, to have this control on every experiment.

In interpreting our results we must primarily define our methods of estimating the glucose content of the blood for it is, of course, problematic as to how accurately the glucose content of blood is determined by reducing, or for that matter, by polariscopic methods. We realize that both of these methods, as employed by us, are inaccurate measurements of the true glucose content. We have assumed, therefore, an arbitrary value in these experiments consisting of the original reducing value minus the reducing value after glycolysis, as representative of the initial glucose content.

The results of nine experiments are shown in Table I and Fig. 1. In Table I the reducing values obtained from the original samples of plasma have been listed in the first column. In three instances we have also obtained corresponding ultrafiltrate reducing and rotatory values, the former generally proving to be higher than the plasma reducing values by about 10 per cent. On this basis we have calculated the approximate ultrafiltrate reducing values of the six other samples and from these figures in turn the glucose content has been estimated.

In Fig. 1 the estimated glucose content of the nine dialysates has been placed upon a diagonal line drawn arbitrarily across the upper half of the graph. The corresponding initial rotatory values, charted as squares, are shown in three instances, and it will be noted that they show fairly close agreement to the glucose content as estimated from the reducing values. Directly below these points the corresponding levorotatory values obtained after glycolysis are shown. It will be seen at a glance that a definite relationship exists between the initial blood sugar values and the rotatory values obtained after glycolysis for the large blood sugar values are associated with large levorotatory values and *vice versa*.

The experiments show therefore that as a result of the glycolytic

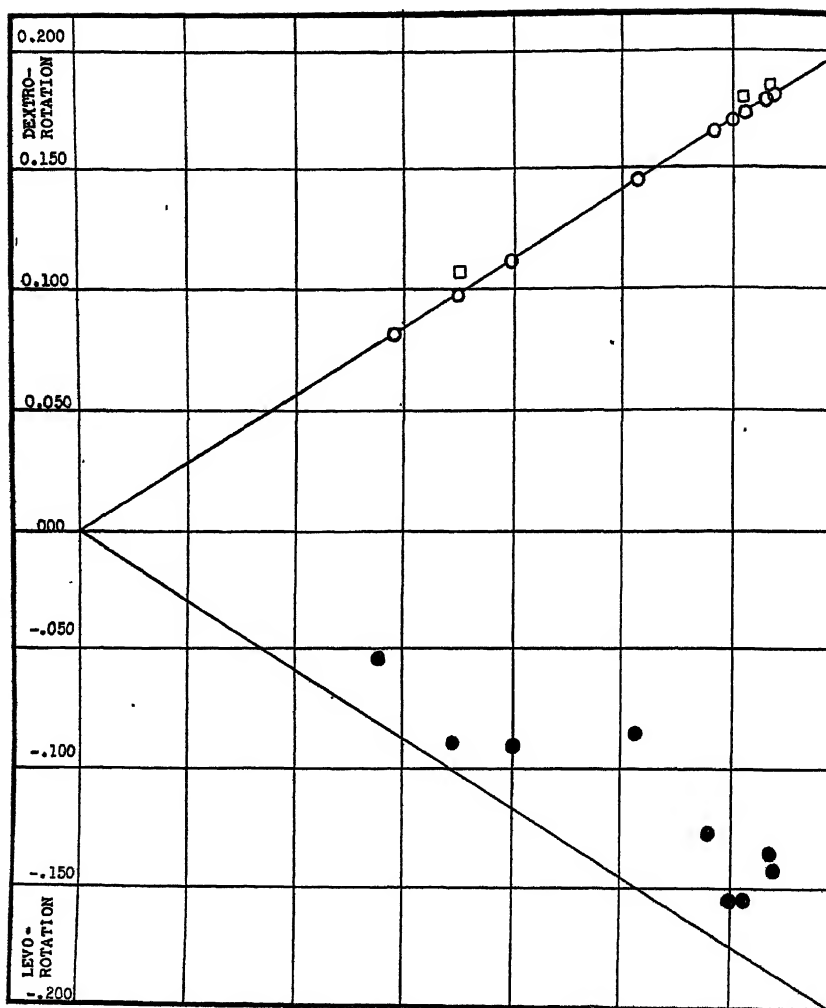


FIG. 1. The relation of initial reducing and rotatory values to the rotatory values after glycolysis in rabbit blood. The clear circles represent the initial estimated glucose content while the squares represent the corresponding rotatory values expressed as glucose. The solid circles represent the postglycolytic rotatory values similarly expressed.

action of whole blood levorotatory substances are formed at the expense of the dextrorotatory substances, and furthermore that we have an interesting and quite unforeseen result, which is shown by the graph, in the symmetry of the distribution of points above and below the zero line. Although one set of points is not exactly a mirror image of the other, in that the levorotation is universally not quite so extensive as the dextrorotation, there is an apparent relationship in the distribution of individual ante- and postglycolytic rotatory values.

Another series of similar readings was then made upon human blood obtained from a group of normal and a group of diabetic individuals. Samples of blood ranging from 50 to 75 cc. were drawn from the arm vein of five normal individuals and five ambulatory diabetic patients who were attending the Diabetic Clinic of the Out Patient Department of the Pennsylvania Hospital.<sup>2</sup>

The diabetic samples were obtained in the morning, the patients having fasted for 12 hours previously. None of the patients was receiving insulin therapy and none of them showed the presence of acetone bodies in the urine at the time the blood sample was obtained.

The postglycolytic rotatory values compared with the corresponding initial values are shown in Fig. 2. As previously pointed out we found the range of blood sugar values in apparently normal rabbits to be greater than in human beings, allowing a wider distribution of points so that the five normal human readings cover such a small area that they give us very limited information. It is interesting to note, however, that these points fall more closely about the line representing the mirror image of the dextrorotatory points than do the other points. The levorotatory values obtained after glycolysis in the diabetic samples fall in a relatively straight line which is fairly well above the line of the mirror image.

We have also charted in Fig. 2 the results obtained in three experiments in which a small quantity of  $\alpha,\beta$ -glucose has been added *in vitro* to a sample of non-diabetic blood so that the glucose content has been elevated to from 220 to 340 mg. per 100 cc. In Fig. 2 it will be seen that the levorotatory values obtained after

<sup>2</sup> We are indebted to Dr. E. S. Dillon for the privilege of studying these patients.

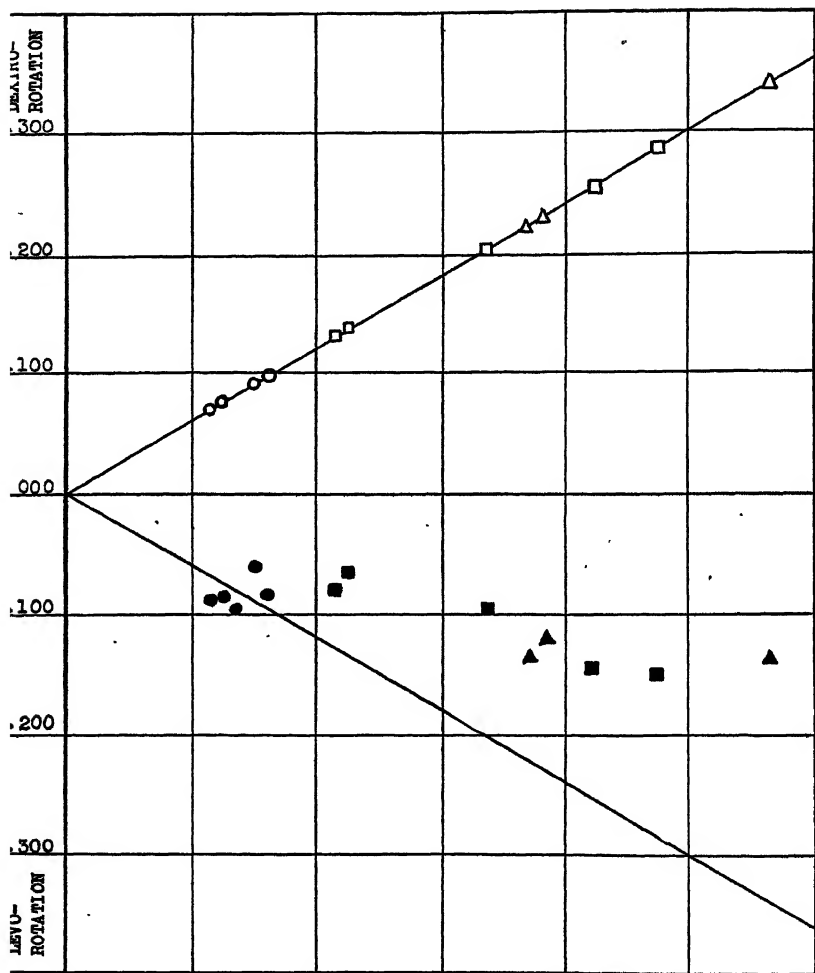


FIG. 2. The relation of initial rotatory values to the rotatory values after glycolysis. The clear circles represent initial rotatory determinations upon n-diabetic samples, the clear squares diabetic samples, and the triangles non-diabetic samples to which glucose has been added. The solid circles, squares, and triangles represent corresponding samples after glycolysis.

glycolysis in these three samples fall approximately in line with those of the diabetics.

We have not attempted to draw conclusions from this small number of experiments except to call attention to the fact that as the blood sugar increased above the normal range, the corresponding degree of levorotation obtained as the result of glycolysis did not increase quite in proportion. Furthermore this phenomenon occurred in diabetic blood in approximately the same manner as it did in non-diabetic blood to which enough glucose had been added to bring the blood sugar level to a similar range. While it is evident that a satisfactory comparison has not been made the experiments do not give us any data which suggest that there is a fundamental difference in this process between normal and diabetic individuals.

A second set of experiments was then devised to determine the relationship which the levorotation in these blood filtrates bore to lactic acid. The experiments were performed in the following manner.

Plasma from samples of defibrinated blood obtained, before, during, and after glycolysis, was filtered and reducing, rotatory, and lactic acid determinations were run upon the resulting filtrates. Readings from samples of diabetic blood and normal blood to which glucose had been added were included. In calculating the amounts of levorotation both before, during, and after glycolysis we have assumed that all of the reducing and dextrorotatory substances present over and above the non-glycolyzable residue represent  $\alpha,\beta$ -glucose. This is perhaps an unwarranted assumption, but as we cannot at present measure any theoretical substances other than  $\alpha,\beta$ -glucose which are both dextrorotatory and at the same time have reducing power we have merely chosen a standard on which to base our calculations. Such theoretical substances, if present, would represent a very small fraction of the total of rotatory and reducing values.

The results are shown in Fig. 3. It will be seen at a glance that levorotation and the lactic acid content of the filtrates show a close relationship and when plotted one against another the points seem to fall roughly along a line.

In the samples of unglycolyzed blood appreciable amounts of

lactic acid were present and not only were we unable to demonstrate the presence of levorotatory substances in these, but apparently a degree of dextrorotation which was slightly greater than

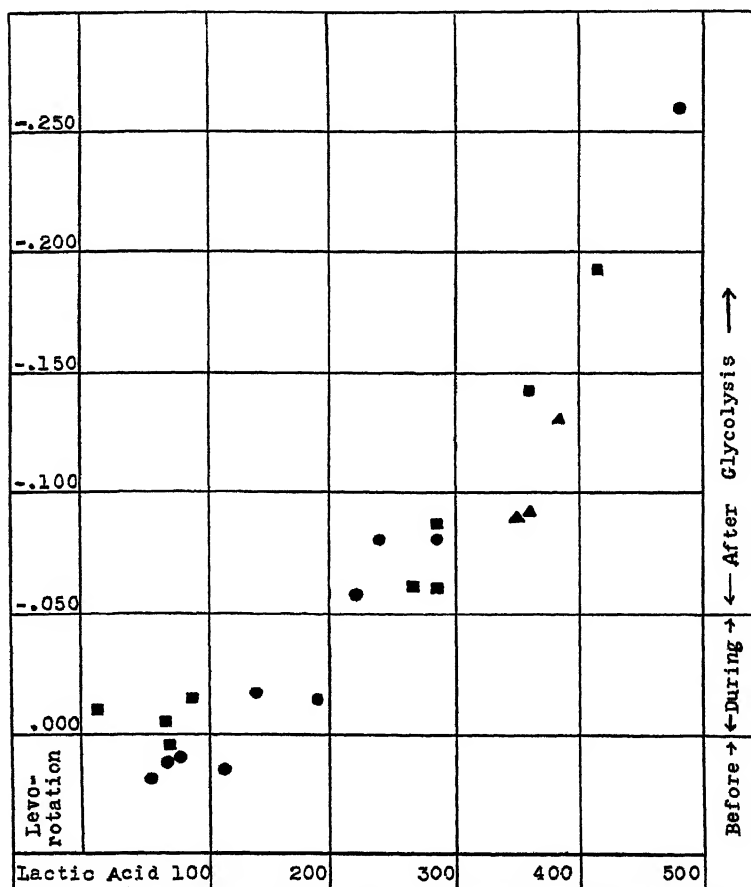


FIG. 3. The relation of levorotation to the lactic acid content of blood. Normal samples are represented by dots, diabetic samples by squares, and added glucose samples by triangles.

that which could be accounted for by  $\alpha, \beta$ -glucose was present. We are unable to explain this discrepancy, which might be due to a variety of factors. What the experiments show, however, is

that as glycolysis proceeds and levorotation and lactic acid increase, the degree of levorotation and the lactic acid content are found to bear a very definite relationship to one another. It would also seem as if the same general relationship existed between levorotation and lactic acid content in the diabetic samples and those to which glucose had been added, as in the normal samples.

The attempt was then made to study the influence which changes in the hydrogen ion concentration exerted upon the optical activity of the plasma filtrates. It is evident that, if the optically active substances other than glucose in which we have been interested are normally represented by the salts of *D*-lactic acid, one would expect that by shifting the pH of these filtrates to the acid side, free *D*-lactic acid would be liberated and this change would find expression in the rotatory values.

It has long been appreciated that the rotatory values noted in blood filtrates are somewhat labile. That they may be influenced by changes in hydrogen ion concentration has also been appreciated. Visscher (5) has called attention to the fact that the optical rotation in filtrates from blood from which the protein has been removed by precipitation may be altered by changes in the hydrogen ion concentration. Anderson and Carruthers (9) have further shown that in alkaline dialysates and filtrates from blood the rotatory values, if expressed in terms of glucose, were low, but on acidifying to a pH of 4 these values rose until they were in practical agreement with the reducing ones. Subsequently on addition of alkali to these solutions, the changes proved to be reversible. In one of their experiments it was noted that a levorotatory value became dextrorotatory on acidification.

In an attempt to study this phenomenon we have performed the following experiments.

After determining the reducing and rotatory values in ultrafiltrate samples of 10 or 15 cc. they were acidified by the addition of *N* HCl, 0.1 cc. at a time. The course of the titration was followed by rough colorimetric determinations of the pH by the spot method, a series of eight Clark and Lubs indicators covering the range of pH 9 to 1 being used. Reducing and rotatory values corrected for dilution were obtained during the course of the titration which was generally carried to a pH of 2. The amount of *N* HCl required to bring the filtrates from an initial pH of 7.6 or 7.8

to 2 varied, due in some measure to an artificial increase in buffer substances as a result of the presence of anticoagulants, but in the usual 15 cc. sample of ultrafiltrate from defibrinated blood, only 0.6 cc. of  $N$  HCl was required. A similar titration with  $N$  NaOH was then generally carried out to obtain another series of readings at or slightly beyond the neutral point. A slightly smaller quan-

TABLE II.

*Effect of pH upon Rotatory Values.*

The values are expressed in terms of gm. of glucose per 100 cc.

	Original reducing value.	Original rotatory value.	Rotatory value on acidification.	Rotatory value on reneutraliza- tion.
Non-glycolyzed samples obtained from miscel- laneous hospital cases.	0.188	+0.193	+0.202	+0.191
	0.151	+0.142	+0.152	+0.145
	0.122	+0.103	+0.122	
	0.120	+0.096	+0.095	
	0.119	+0.100	+0.135	
	0.113	+0.107	+0.113	+0.101
	0.112	+0.115	+0.153	+0.115
	0.109	+0.038	+0.082	+0.065
	0.098	+0.087	+0.115	+0.082
	0.086	+0.084	+0.099	
	0.084	+0.080	+0.101	+0.077
	0.082	+0.071	+0.088	
Samples in which glycoly- sis had progressed.	0.134	-0.087	+0.044	-0.064
	0.056	-0.051	-0.029	-0.046
Samples obtained post mortem.	0.110	+0.013	+0.090	-0.001
	0.067	-0.128	-0.031	-0.121
	0.063	-0.107	+0.039	-0.073
	0.016	-0.052	-0.031	-0.055

tity of alkali was required than of acid, the difference agreeing well with the  $CO_2$  content of the ultrafiltrates.

In the course of our work it immediately became apparent that the effect upon the rotatory values produced by altering the pH of these solutions was dependent upon the degree of levorotation present. In other words the largest shifts were obtained in those specimens in which the levorotatory values proved to be the largest and these in turn were found to be particularly prominent in



samples of blood in which glycolysis had progressed and in post-mortem samples.

The results of a series of titrations are shown in Table II. In Table II the samples have been divided into three groups on the basis of the degree of glycolysis which had taken place. In the first series we included samples obtained from a group of hospital patients in which the blood sugar values fell within the normal range. In the second we included samples in which glycolysis had been allowed to proceed and in the third, samples which had been obtained within a few hours or minutes after death. In the first two columns of Table II we may compare the relationship which the initial rotatory values bear to the reducing ones, both of which are expressed in terms of glucose. In the first group of blood samples the rotatory values are generally less than the reducing ones by about 10 or 20 per cent. In the second and third groups we find a much larger discrepancy between these two values due to the presence of an excess of levorotatory substances. In the second and third columns of Table II we may compare the changes in rotation produced by bringing the filtrates to a pH of about 2, and we note that, as a result of acidifying, the rotatory values always rise and may approximate the reducing values; in a few instances they may even be somewhat greater. This change in rotation is far more pronounced in the samples of blood in which glycolysis had proceeded and in one instance we note a shift which represents the equivalent of 0.130 gm. of glucose. In two instances the values change from levorotatory to dextrorotatory. However, the shifts obtained in the samples of unglycolyzed blood are of sufficient similarity to those of the other groups to suggest that the substance either partially or wholly responsible for these changes (presumably lactic acid) which is present in normal blood, may be of the same general type as that produced by the glycolytic action of whole blood.

Finally in an attempt to obtain more data with regard to the optical properties of the lactic acid formed by the glycolytic action of whole blood, a small quantity of this substance was isolated from the blood in the form of its zinc salt.

The procedure employed was as follows: Several samples of defibrinated blood were allowed to glycolyze for 24 hours and from them, plasma filtrates were obtained either by the method outlined above or by the precipitation of the proteins with sodium tungstate and sulfuric acid. In the latter case

the filtrate was reconcentrated *in vacuo*. The filtrates were then transferred to an extractor similar to that described by Clausen (10) and extracted with ether for 1 to 2 hours. This preliminary extraction served to remove any substances which might be extracted by ether from a neutral or slightly alkaline plasma filtrate. Lactic acid determinations made upon the filtrates both before and after this extraction, showed no change. They were then acidified with sufficient sulfuric acid to bring the pH to a value less than 2. This solution was extracted with a fresh portion of ether for about 5 hours and in most instances a yield of about 75 per cent of the free lactic acid from the filtrate was obtained in the ether extract, which was evaporated and the residue taken up in a small quantity of distilled water. To this a suspension of zinc carbonate was added and the resulting solution was allowed to stand overnight. The excess zinc carbonate was then



FIG. 4. Photograph of the crystals of zinc lactate isolated from blood.

filtered while warm leaving a clear solution which on evaporation to dryness yielded characteristic crystals.

The crystals from three blood samples were mixed together and then redissolved in water which was acidified to a pH of less than 2 with sulfuric acid. This solution was reextracted for a period of 15 hours with ether. The residue from the ether extract was taken up in 15 cc. of water and was found to contain about 0.600 gm. of lactic acid per 100 cc., presumably in its free state.  $\text{ZnCO}_3$  was then added as before, and the solution containing zinc lactate was partially evaporated over the water bath, filtered, brought up to the original volume of 15 cc., and read in the polariscope. Subsequently on the addition of a few drops of NaOH, filtering off the precipitate of  $\text{Zn(OH)}_2$ , and titrating back to neutrality with HCl, the solu-

tion, this time presumably containing sodium lactate, was also read in the polariscope. Another reading was then obtained on reacidification.

Finally a third extraction with ether was made and the resulting solution to which zinc carbonate had been added was evaporated to dryness at room temperature for the study of the crystals which appeared as fine needles. A photograph of the crystals obtained is shown in Fig. 4.

In Table III we have tabulated the results of our polariscopic studies upon the solution, from which the crystals of zinc lactate shown in Fig. 4 were isolated. The readings have been arbitrarily expressed in terms of glucose for purposes of comparison with the previous studies upon the filtrates. In the original aqueous solu-

TABLE III.

*Rotatory Values of Isolated d-Lactic Acid Solution.*

For purposes of comparison with the figures obtained in the blood filtrates the rotatory values are expressed in terms of gm. of glucose per 100 cc.

	pH	Rotatory value.	Lactic acid, gm. per 100 cc.
Original aqueous solution.....	3.0	0.00	0.599
After addition of $\text{ZnCO}_3$ .....	6.4	-0.161	
“ “ “ NaOH.....	7.2	-0.151*	
“ acidification with HCl.....	2.0	+0.007	

\* In the conversion of zinc lactate to sodium lactate the experiment was not exactly quantitative.

tion containing free lactic acid in a slightly higher concentration than that which we have studied in our plasma filtrates, we were unable to demonstrate any optical activity. With the formation of both zinc and sodium lactate very appreciable degrees of levorotation were observed which disappeared in the latter instance on acidification, recalling the optical behavior which we have repeatedly noted in the blood filtrates.

The attempt was then made to determine the specific rotation of the small sample of zinc lactate which we had isolated from blood. The procedure presented difficulties in that owing to the small amount of crystals available it was necessary to make our observations upon a very dilute solution and the number of standard determinations of the specific rotation of zinc lactate at this dilution is apparently limited.

Our total yield of zinc lactate crystals proved to be 51 mg. of which 6 mg. were lost on heating to  $110^{\circ}$ . This gave a percentage of water of 11.7 as compared with the theoretical percentage of 12.9. The total yield of crystals was dissolved in 5.18 cc. of water, giving a 0.81 per cent solution, based on the anhydrous salt. With the yellow light, a tube 1 dm. in length being used,  $[\alpha]_D = -9.7^{\circ}$  for this solution. This may be compared with the results which we have calculated from the figures of Jungfleisch and Godchot (11) giving  $[\alpha]_D = -9.14^{\circ}$  for a solution of 1.25 per cent and  $-10.99^{\circ}$  for 0.512 per cent, or those of Purdy and Walker (12) giving  $-10.29^{\circ}$  for a solution of 1.215 per cent. It is evident, however, that further work upon the determination of the optical properties of the different salts of *d*-lactic acid particularly in dilute solution is necessary.

#### DISCUSSION.

In brief, therefore, the findings point to the fact that *d*-lactic acid, which exists in the blood in the form of a levorotatory salt, may be considered as one of the important optically active substances of the blood, and after the glycolytic action of whole blood has been allowed to progress, lactates or related substances may accumulate in sufficiently large quantities to enable them to be easily measured by the polariscope in an unconcentrated sample of blood filtrate. This phenomenon has been observed in the blood of rabbits, normal individuals, and mildly diabetic individuals. We do not maintain, however, that the salts of *d*-lactic acid are the only demonstrable optically active substances other than glucose in normal blood, either before or after glycolysis, but it would appear that they are among the most prominent.

Of some importance is the bearing which these observations have upon studies that have been made upon the rotatory and reducing determinations of the blood in which these properties have been expressed in terms of  $\alpha,\beta$ -glucose. Such studies have been discussed in our previous papers (1, 2). They include those of Winter and Smith (13) and Lundsgaard and Holbøll (14), who have suggested that the discrepancies between reducing and rotatory determinations in normal blood may be due to the fact that the glucose of normal blood is of lower specific rotation than that of  $\alpha,\beta$ -glucose. The present study points to the fact that *d*-lactic

acid in the form of one of its salts is one of the important substances which may be responsible for the production of discrepancies between the reducing and rotatory properties of the blood if we attempt to express them both in terms of glucose.

In conclusion we wish to express our appreciation to Dr. J. H. Austin for his helpful criticisms in the course of the experiments, and to Dr. J. T. Bauer for photographing the crystals.

#### CONCLUSIONS.

A study has been made on the levorotatory substances which are formed at the expense of glucose as a result of the glycolytic action of animal and human blood and evidence has been brought forward to favor the assumption that these levorotatory substances represent in large measure the salts of *d*-lactic acid.

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**THE INFLUENCE OF FASTING AND CREATINE FEEDING  
UPON THE CREATINE CONTENT OF THE TISSUES  
AND BLOOD OF THE WHITE RAT.\***

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The recent review of the literature on creatine and creatinine by Hunter (1) shows that comparatively few investigations have been made with respect to the factors involved in the storage of creatine in the body tissues, particularly during fasting.

Mendel and Rose (2, *a*) have presented evidence to show that "there occurs an increase in the percentage of creatine in the muscles of rabbits and fowl during inanition." According to Myers and Fine (3), "The creatine content of rabbit muscle is relatively increased in the early part of starvation, but decreased at the close of the starvation, owing to the great loss of creatine in the urine during this period." Evidence both for and against these ideas has been presented by other workers (1). It may be said that the majority of the evidence points towards a decrease in the creatine content of muscle as a result either of inanition or malnutrition.

The investigations recorded in this paper were begun in view of the divergent results obtained by workers interested in the relationship of the creatine content of muscle to inanition. In order to study this problem effectively, a large number of white rats were employed.

The unexpected results obtained led us to try further experiments in order to test the present theories concerning the storage of creatine in the body. The present investigation deals with the

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creatine concentration of muscle, liver, kidneys, testes, heart, and brain as influenced by (a) loss of body weight due to fasting, (b) re-feeding creatine and control diets after prolonged fasting, and (c) feeding creatine diets to normal rats with the consequent effect of fasting and substitution of a control diet. In addition blood studies were made under the various conditions noted above.

#### EXPERIMENTAL.

The albino rat served as the experimental subject. These animals were maintained on a stock diet.<sup>1</sup> Recrystallized creatine was added in proper amounts to the above food mixture so as to make a 5 and 10 per cent creatine diet. In all cases, the animals were placed on the control diet for at least a week preceding any experimental work. Those animals fed on experimental diets were allowed food up to the time of killing. During the period of fasting, the rats were placed in individual double bottom mesh cages to avoid coprophagy. Water was always available.

In order to obtain the tissues and blood for analysis, the animals were stunned by a sharp blow behind the head and were immediately decapitated. The blood was allowed to drain into a small beaker containing powdered potassium oxalate. The various tissues to be analyzed were promptly removed and dissected free from all visible fat. The muscles of the hind legs were ground in a small meat grinder and samples were taken for creatine analysis and total solids. The remaining tissues were cut into small pieces with scissors and were analyzed for creatine. The analysis for creatine (total creatinine) was made by the method of Rose, Helmer, and Chanutin (4). Every effort was made to treat the tissues with sulfuric acid within a few minutes after the death of the animal. The non-protein nitrogen, total creatinine, and pre-formed creatinine were determined on the tungstic acid filtrate according to the method of Folin and Wu (5). The reducing sugar was determined by the method of Benedict (6).

<sup>1</sup> Whole wheat flour two-thirds, dried whole milk one-third, 1 per cent of the weight of the wheat as sodium chloride, and 1 per cent of the weight of the wheat as calcium carbonate. Lettuce was fed three times a week and water was given *ad libitum*.



*Effect of Fasting upon Creatine Content of Various Tissues.*

Because of the small amount of tissue generally available, it was possible to determine the total solids only of the muscle. All figures for the creatine content of muscle were changed to a basis of the uniform total solid content of 25.1 per cent, obtained by averaging the results on twenty-three control animals. The importance of this conversion is apparent upon examination of the data dealing with muscle creatine. It is noted in Table I that the

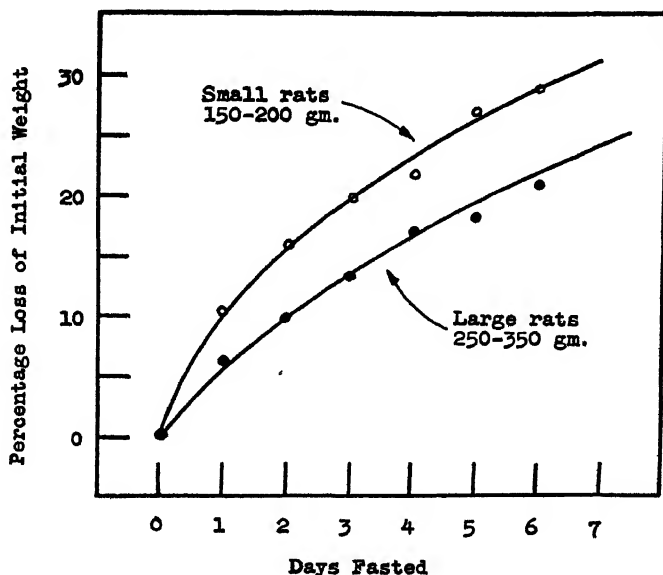


CHART 1. Each point on these curves represents fifteen or more rats.

total solids increase during the early stages of fasting and decrease below the normal level as fasting continues. These findings confirm the results obtained by other workers (2, a, 3).

It is demonstrated in Chart 1 that the percentage loss of weight over a given period of time is dependent upon the initial weight of the animal. It seems, therefore, that the percentage loss of body weight serves as a better standard for comparison than the length of time of fasting in studying the changes in the creatine content of tissues.

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TABLE I.

*Influence of Fasting upon Creatine Content of Rat Tissues.*

Figures expressed in per cent.

	Days fasted.	Weight lost.	Liver.	Kidneys.	Testes.	Heart.	Brain.	Muscle.	Total solids.	Muscle, corrected.
Control group.										
Minimum.....			0.036	0.043	0.265	0.168	0.145	0.418	23.1	
Maximum.....			0.053	0.071	0.324	0.263	0.168	0.478	27.7	
Average.....			0.041	0.055	0.299	0.217	0.154	0.454	25.1	
No. of rats.....			12	14	15	21	17	22	23	
Group I (0-10 per cent body weight lost).										
Minimum.....	1	4.9	0.032	0.060	0.268	0.177	0.150	0.453	24.1	0.451
Maximum.....	4	10.0	0.040	0.094	0.342	0.240	0.184	0.491	26.2	0.491
Average.....	1.5	8.0	0.036	0.077	0.295	0.201	0.164	0.472	25.3	0.468
No. of rats.....	8		6	8	7	8	5	7	8	7
Group II (10-20 per cent body weight lost).										
Minimum.....	2	10.7	0.031	0.058	0.279	0.159	0.138	0.417	24.2	0.422
Maximum.....	5	18.0	0.060	0.113	0.389	0.241	0.173	0.489	27.3	0.554
Average.....	3.1	15.3	0.039	0.077	0.318	0.197	0.155	0.498	25.4	0.491
No. of rats.....	18		16	15	14	18	15	18	18	18
Group III (20-30 per cent body weight lost).										
Minimum.....	3	20.8	0.031	0.065	0.301	0.182	0.148	0.434	24.2	0.430
Maximum.....	9	28.8	0.055	0.100	0.364	0.254	0.182	0.555	27.2	0.581
Average.....	5.0	24.3	0.040	0.080	0.319	0.215	0.162	0.503	25.1	0.503
No. of rats.....			9	20	15	20	8	19	20	19
Group IV (30-40 per cent body weight lost).										
Minimum.....	4	30.0	0.037	0.069	0.214	0.181	0.145	0.480	23.4	0.470
Maximum.....	11	43.7	0.060	0.097	0.358	0.287	0.191	0.589	25.7	0.626
Average.....	7.2	33.8	0.044	0.083	0.314	0.230	0.163	0.561	24.3	0.570
No. of rats.....			12	18	16	17	11	18	19	18

The data on the influence of fasting on the creatine content of various tissues are summarized in Table I. It is noted that of the tissues analyzed, the muscle shows the greatest change in its creatine content as a result of fasting. The muscle creatine of the animals in Group I, which represents the early stages of fasting, shows only a slight average increase of 3.9 per cent over the control group. In Groups II and III, the muscle creatine content is increased 8.1 and 10.8 per cent respectively. The average creatine concentration of the muscle of the animals in Group IV (terminal stage) shows a marked increase of 25.6 per cent above normal. Practically all rats die of fasting after 30 to 40 per cent of the

TABLE II.

*Comparison between Loss of Tissue Weight and Creatine Concentration as Result of Acute Inanition (Loss of Body Weight, 33 Per Cent).*

	Loss of tissue weight.	Change in creatine concentration.
	<i>per cent</i>	<i>per cent</i>
Liver.*.....	-58.0	+7.3
Kidneys.....	-26.0	+51.0
Testes.....	-30.0	+3.0
Heart.....	-28.0	+6.0
Brain.....	-5.0	+5.8
Muscle.....	-31.0	+25.6

\* These figures were compiled by Jackson (11).

body weight has been lost. As a rule, an animal in this group was not killed until it was very weak and on the verge of death.

Not only do these results indicate that the creatine content of muscle progressively increases during fasting, but they show that a relatively sharp increase occurs shortly before death.

In Table II it will be noted that there is no absolute relationship between loss of weight and creatine concentration of tissues during the extreme stages of fasting. The creatine content of the kidneys and muscle is definitely increased during this experimental condition. The increased creatine content of the kidneys probably has no direct metabolic significance, but is due to the resulting creatinuria of fasting. The results obtained for the remaining tissues cannot be considered as significant, since a variation in total solids may account for the slight rise in creatine obtained. This same

argument may be used to explain the small variations in the creatine content of the heart and testes during the course of fasting (Table I).

*Refeeding a 10 Per Cent Creatine and Control Diet after Prolonged Inanition.*

In the foregoing data, it was demonstrated that the greatest increase in the creatine concentration of muscle occurred just before death. It seemed probable that the refeeding of a creatine-containing diet at this stage might result in a cumulative effect. As may be seen in Table III, the results obtained are striking. The creatine of the muscle increases to a level appreciably higher (4.8 per cent) than the average maximum concentration obtained during fasting. We believe this creatine concentration in muscle (30.4 per cent above normal) to be higher than any creatine storage reported in the literature. Despite continued creatine feeding, the muscle creatine content drops during the 2nd and 3rd days. Evidently the creatine reservoir can retain less and less creatine as the animal regains its weight. The creatine concentration after 3 days of creatine refeeding is about the same as that obtained by feeding the same diet to control animals (Table V).

A further consideration of the data in Table III shows that the ingestion of creatine after prolonged fasting has also caused a decided increase in the creatine concentration of other tissues. This is particularly striking in the case of the liver in which there is an increase of about 1000 per cent after 1 day's creatine feeding. During the 2nd and 3rd days, there is a gradual decrease. The increase in the creatine content of the kidneys is marked (360 per cent) and remains fairly constant for the duration of the experiment. A maximum increase of about 42 per cent above the highest figure obtained during fasting is noted in the creatine concentration of the heart. The creatine content of the testes and brain, however, is practically the same as in animals showing a similar loss of weight due to inanition.

The refeeding of a control diet after fasting (Table IV) throws further light on the tendency of the creatine reservoir to return to the normal level. The creatine of the muscle drops after 1 day's refeeding, and continues to decrease proportionately as the body weight increases. The analyses of the kidneys seem to indicate

that creatinuria still persists during the 1st day of refeeding. The creatine content drops to normal on the 2nd day and remains constant at this level during the period of the experiment. Although the remaining tissues seem to return to normal, no definite conclusion can be drawn because of the few analyses.

TABLE III.

*Effect of Creatine Refeeding after Prolonged Fasting.*

Figures expressed in per cent.

	Weight lost after:		Liver.	Kidneys.	Testes.	Heart.	Brain.	Muscle.	Total solids.	Muscle, corrected.
	Fasting.	Refeeding.								
Refed 1 day.										
Minimum.....	26.7	16.8	0.396	0.214	0.275	0.258	0.155	0.492	23.0	0.527
Maximum.....	34.2	30.8	0.504	0.297	0.355	0.328	0.188	0.640	25.6	0.656
Average.....	30.6	21.6	0.432	0.254	0.313	0.303	0.168	0.567	24.0	0.592
No. of rats.....	6	6	6	6	5	5	5	6	6	6
Refed 2 days.										
Minimum.....	25.9	12.1	0.305	0.214	0.296	0.313	0.170	0.454	22.0	0.482
Maximum.....	31.6	26.4	0.412	0.297	0.333	0.337	0.171	0.541	24.6	0.591
Average.....	27.8	18.0	0.384	0.247	0.315	0.327	0.171	0.516	23.5	0.550
No. of rats.....	7	7	7	6	2	3	2	7	7	7
Refed 3 days.										
Minimum.....	24.3	11.0	0.211	0.200	0.306	0.252	0.153	0.469	23.1	0.510
Maximum.....	36.0	23.8	0.358	0.271	0.313	0.292	0.160	0.544	24.6	0.561
Average.....	28.7	17.9	0.279	0.225	0.309	0.273	0.157	0.508	23.8	0.535
No. of rats.....	6	6	6	3	3	3	3	6	6	6

In both these series of experiments, the water content of the muscle presents an interesting picture. Beginning with the 1st day and continuing throughout the remainder of the experimental period, there is a rise in the water content of the muscle which is definite and striking. This result would seem to indicate that an excessive storage of water, as judged from muscle, plays an important rôle in the regaining of body weight after prolonged fasting.

*Effect of Feeding 5 and 10 Per Cent Creatine Diets on Tissues of Normal Young and Adult Animals.*

The data in Tables V to VII throw further light on the ability of the tissues to store creatine. It is noted that there is a marked

TABLE IV.

*Effect of Refeeding Control Diet after Prolonged Fasting.*

Figures expressed in per cent.

	Weight lost after:		Liver.	Kidneys.	Testes.	Heart.	Brain.	Muscle.	Total solids.	Muscle, corrected.
	Fasting.	Refeeding.								
Refed 1 day.										
Minimum.....	26.6	22.2	0.044	0.071	0.306	0.239	0.145	0.469	23.1	0.505
Maximum.....	35.4	30.4	0.068	0.098	0.309	0.264		0.556	24.4	0.601
Average.....	29.7	24.8	0.057	0.085	0.308	0.252	0.145	0.518	23.5	0.554
No. of rats.....	7	7	5	6	2	2	1	7	7	7
Refed 2 days.										
Minimum.....	29.4	19.0	0.057	0.045	0.331	0.237		0.461	23.2	0.500
Maximum.....	32.5	25.6				0.248		0.517	23.8	0.559
Average.....	32.7	23.2	0.057	0.045	0.331	0.243		0.498	23.4	0.535
No. of rats.....	3	3	1	1	1	2		3	3	3
Refed 4 days.										
Minimum.....	33.4	16.3		0.042	0.283	0.233		0.456	22.8	0.486
Maximum.....	35.3	19.5			0.286	0.236		0.465	23.6	0.510
Average.....	34.3	17.9		0.042	0.285	0.235		0.460	23.2	0.498
No. of rats.....	2	2		1	2	2		2	2	2
Refed 6 days.										
Rat 105.....	31.6	9.8			0.336	0.235		0.469	23.6	0.498

increase in the creatine content of the liver, kidneys, heart, and muscle after 1 day's feeding (Table V). Continued feeding of creatine is accompanied by a definite decrease in all of the tissues mentioned above. This picture seems to be typical of the various experiments of this nature. Under these experimental conditions

there is a maximum average increase of 17.5 per cent for the muscle, 550 per cent for the liver, 330 per cent for the kidneys, and 30 per cent for the heart. It is clear from consideration of this and the previous experiment that the creatine reservoirs are capable of even

TABLE V.

*Effect of Feeding 10 Per Cent Creatine Diet on Tissues of Normal Rats.*

Figures expressed in per cent.

	Weight, gm.	Liver.	Kidneys.	Testes.	Heart.	Brain.	Muscle.	Total solids.	Muscle, corrected.
Fed 1 day.									
Minimum.....	167	0.195	0.156	0.253	0.249	0.154	0.486	23.5	0.495
Maximum.....	322	0.294	0.254	0.324	0.296	0.178	0.580	25.1	0.594
Average.....		0.267	0.221	0.289	0.261	0.165	0.516	24.3	0.533
No. of rats.....		8	7	8	5	6	8	8	8
Fed 2 days.									
Minimum.....	170	0.167	0.152	0.260	0.266	0.162	0.438	24.2	0.465
Maximum.....	312	0.330	0.309	0.295	0.300	0.188	0.513	24.7	0.529
Average.....		0.244	0.237	0.277	0.282	0.179	0.472	24.3	0.490
No. of rats.....		7	7	7	5	5	7	7	7
Fed 3 days.									
Minimum.....	240	0.220	0.099				0.487	23.1	0.515
Maximum.....	318	0.275	0.187				0.498	24.0	0.530
Average.....		0.241	0.152				0.493	23.6	0.522
No. of rats.....		3	4				4	4	4
Fed 4 days.									
Minimum.....	171	0.146	0.107	0.308	0.229	0.157	0.450	24.3	0.461
Maximum.....	318	0.306	0.252	0.344	0.250	0.189	0.519	24.5	0.535
Average.....		0.235	0.185	0.322	0.238	0.174	0.491	24.4	0.506
No. of rats.....		6	6	3	4	4	6	6	6

greater storage capacities, particularly in the case of the muscle and liver. It is to be noted that the water content of the muscle in this series is increased as a result of creatine feeding.

Feeding experiments with a 5 per cent creatine diet for 19 days are summarized in Table VI. We believe these figures represent

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a state of creatine equilibrium in the organism. It is noted that the creatine content of various tissues is increased and also that the total solids for muscle are normal.

TABLE VI.  
*Effect of Feeding 5 Per Cent Creatine Diet for 19 Days on Tissues of Normal Rats.*

Figures expressed in per cent.

Rat No.	Weight.	Liver.	Kidneys.	Testes.	Heart.	Brain.	Muscle.	Total solids.	Muscle, corrected.
	gm.								
114	212	0.158	0.136	0.348	0.253	0.170	0.543	25.3	0.539
115	177	0.129	0.119		0.268	0.181	0.569	25.4	0.560
116	205	0.301	0.277	0.330	0.275	0.177	0.545	25.2	0.544
		0.199	0.177	0.339	0.265	0.176	0.552	25.3	0.548

TABLE VII.  
*Effect of Feeding 10 Per Cent Creatine Diet on Liver and Muscle of Young Rats.*  
Figures expressed in per cent.

	Age, days.	Weight, gm.	Liver.	Muscle.	Total solids.	Muscle, corrected.
Controls.						
Minimum.....	44	80	0.046	0.382	24.2	0.396
Maximum.....	47	110	0.080	0.464	26.0	0.447
Average.....			0.047	0.415	25.1	0.415
No. of rats.....			6	6	6	6
Fed 1 day.						
Minimum.....	44	64	0.262	0.461	22.7	0.496
Maximum.....	45	129	0.418	0.499	25.0	0.528
Average.....			0.323	0.480	23.8	0.506
No. of rats.....			4	5	5	5
Fed 2 days.						
Minimum.....	46	106	0.109	0.462	23.1	0.485
Maximum.....	48	141	0.153	0.494	23.9	0.529
Average.....			0.133	0.481	23.6	0.511
No. of rats.....			3	3	3	3



The creatine content of the liver and muscle of young rats fed a creatine-containing diet is decidedly increased (Table VII). The muscle of these young animals shows an increase of 22 per cent, an increase definitely higher than that obtained with adult rats under the same conditions. Possibly the low creatine content of the normal muscle, characteristic of young animals, allows for a greater creatine capacity. The liver creatine is increased as a result of the 1st day's creatine ingestion, but the succeeding day's figures show a marked drop. In these experiments we are again confronted with the decrease in muscle total solids.

TABLE VIII.

*Effect of Discontinuing Creatine Feeding on Tissues of Normal Rats.*

Figures expressed in per cent.

Rat No.	Weight, gm.	Fed creatine, days.	Liver.	Kidneys.	Testes.	Heart.	Brain.	Muscle.	Total solids.	Muscle, corrected.
Fasted 1 day.										
160	167	7	0.042	0.088	0.274	0.229	0.144	0.473	24.7	0.481
161	188	7	0.044	0.071	0.268	0.236	0.143	0.505	23.7	0.534
162	149	7	0.039	0.073	0.298	0.285	0.151	0.476	23.9	0.500
219	196	10	0.032	0.065	0.315	0.299	0.183	0.553	24.3	0.571
			0.039	0.074	0.289	0.263	0.156	0.504	24.2	0.523
Control diet substituted for 1 day.										
205	297	3	0.045	0.050				0.512	23.9	0.536
206	302	3	0.063	0.060				0.503	24.6	0.513
			0.054	0.055				0.508	24.3	0.525

*Effect of Discontinuation of Creatine Diets on Tissue Creatine in Normal Rats.*

After adult rats were fed on a 10 per cent creatine diet for a sufficient length of time to assure an adequate storage of excess creatine in the tissues, food was withdrawn in one series and a control diet substituted in another (Table VIII). The sudden withdrawal of creatine causes a rapid disappearance of the excess creatine from the liver and kidneys during the 1st day. The muscle, however, shows only a very small loss of creatine under these conditions.

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TABLE IX.

*Effect of Fasting on Blood of Rats.*

Figures expressed in mg. per 100 cc. of blood.

	Non-protein N.	Sugar.	Total creati- nine.	Pre- formed creati- nine.	Creatine expressed as creati- nine.
Control group.					
Minimum.....	37	115	5.1	1.1	4.3
Maximum.....	51	154	6.7	2.1	
Average.....	43	136	5.7	1.4	
No. of rats.....	7	7	7	5	
Group I.*					
Minimum.....	35	86	6.0	1.4	5.1
Maximum.....	48	103	7.1	1.7	
Average.....	41	93	6.6	1.5	
No. of rats.....	4	4	3	3	
Group II.					
Minimum.....	37	71	5.7	1.3	4.9
Maximum.....	55	112	7.7	1.4	
Average.....	46	94	6.3	1.4	
No. of rats.....	10	10	7	3	
Group III.					
Minimum.....	42	78	5.1	1.4	4.9
Maximum.....	71	185	7.7	1.7	
Average.....	51	121	6.4	1.5	
No. of rats.....	8	8	8	3	
Group IV.					
Minimum.....	32	104	5.9	1.4	4.5
Maximum.....	67	127	6.2	1.7	
Average.....	48	114	6.1	1.6	
No. of rats.....	6	8	3	2	

\* Refer to Table I.

*Effects of Fasting upon the Blood.*

The data showing the effect of fasting on several constituents of the blood are given in Table IX.

In these experiments the non-protein nitrogen of the blood shows very little change even after the extreme stages of fasting are

TABLE X.

*Effect of 10 Per Cent Creatine Ingestion on Blood of Rats.*

Figures expressed in mg. per 100 cc. of blood.

	Non-protein N.	Sugar.	Total creati- nine.	Pre- formed creati- nine.	Creatine expressed as creati- nine.
Fed 1 day.					
Minimum.....	41	125	19	1.4	39.1
Maximum.....	61	147	72	2.3	
Average.....	49	134	41	1.9	
No. of rats.....	6	6	5	5	
Fed 2 days.					
Minimum.....	47	124	19	1.4	23.2
Maximum.....	55	146	31	2.3	
Average.....	51	134	25	1.8	
No. of rats.....	5	4	4	5	
Fed 3 days.					
Minimum.....	39	132	12	1.8	14.9
Maximum.....	40	134	22	2.3	
Average.....	40	133	17	2.1	
No. of rats.....	2	2	2	2	
Fed 4 days.					
Minimum.....		144	24	1.7	23.3
Maximum.....		151	25		
Average.....		148	25	1.7	
No. of rats.....		2	2	1	

reached. Although there is an average maximum increase of about 15 per cent, this cannot be taken at its face value because of the individual variations within each group. There is evidence that a few of the animals show a distinct rise in non-protein nitrogen toward the later stages of fasting. These data are not in

strict accord with those of Morgulis and Edwards (7), who found an unmistakable rise in non-protein nitrogen at the terminal stage of fasting in dogs.

Morgulis and Edwards noted further a fall in the blood sugar values of the dog in the early stages of fasting, followed by a rise above normal with further progress of the fast. A similar curve was obtained in these experiments, but the blood sugar concentration never reached the normal level during any stage of fasting.

Although our analyses for creatine and creatinine are few in number, we must conclude from these data that these two components of the blood are not affected as a result of fasting. This is rather surprising in the light of the changes that were found to have taken place in the creatine concentration of muscle and kidneys. It is certain that any marked changes in the blood creatine and creatinine would have been noted despite the paucity of the material.

*Effect of Feeding a 10 Per Cent Creatine Diet upon the Blood.*

The blood analyses in rats fed a diet containing 10 per cent creatine are summarized in Table X. It will be observed that the non-protein nitrogen is increased slightly under these conditions. The sugar concentration remains normal. According to Hill (8), the oral administration of creatine causes a decrease in the blood sugar. Creatine ingestion in the rat apparently does not have the same effect on blood sugar as is encountered in dog and man. As one might expect, the blood creatine has been markedly increased. The preformed creatinine of the blood in these animals is definitely higher than in the controls. This finding confirms the idea that ingestion of creatine in sufficient quantities is accompanied by an increase of creatinine in the blood and urine.

*Effect of Refeeding Control and Creatine Diets upon the Blood.*

The outstanding result of refeeding a control diet after prolonged fasting is the unquestionable increase in the total creatinine. This increase may be due to the decrease in the creatine content of muscle, with a consequent liberation of creatine into the blood stream. The refeeding of creatine causes an increase, not only in the total creatinine, but also in the blood sugar (Table XI).

Although creatine is known to be a weak reducing agent, it cannot be responsible for this increased blood sugar.

In these blood studies the constancy of creatine and creatinine during fasting should be emphasized. The most marked changes in the blood creatine and creatinine are obtained after creatine ingestion. The results of the determination of blood non-protein nitrogen and sugar are not significant in our consideration of creatine and creatinine metabolism.

TABLE XI.

*Effect of Refeeding Creatine and Control Diets after Prolonged Fasting.*

Figures expressed in mg. per 100 cc. of blood.

	Non-protein N.	Sugar.	Total creati- nine.	Pre- formed cresti- nine.	Creatine expressed as creati- nine.
Refed normal diet 1 day.					
Minimum.....	38	95	6.1	1.4	6.8
Maximum.....	58	133	10.1	1.9	
Average.....	48	111	8.4	1.6	
No. of rats.....	5	5	5	4	
Refed 10 per cent creatine diet 1 day.					
Minimum.....	40	120	22		
Maximum.....	58	145	62		
Average.....	49	134	34		
No. of rats.....	4	5	5		

#### DISCUSSION.

The chief points of interest in the foregoing studies have been the bearing of the results upon creatine storage and metabolism in the organism. It has been demonstrated that the ability to store creatine in the tissues, particularly in muscle, is a relative one, and is dependent upon the experimental conditions. Furthermore, a comparison of the creatine storage under varying conditions of nutrition has been made, which serves as an excellent basis for discussing creatine metabolism.

The literature concerning the relationships of creatine metabolism to tissues, limited for the most part to muscle, deals with experimental methods which are both direct and indirect. Efforts

to relate the creatinuria of fasting and the creatine content of muscle have led to a number of theories concerning certain phases of creatine metabolism. Hunter (1) has summarized the ideas offered by many workers to explain the origin of urinary creatine during fasting, as follows: (1) Protein catabolism causes a decrease in the power of the body to dehydrate creatine (Cathcart). (2) Urinary creatine represents preexisting creatine released from disintegrating muscle, and is excreted without change (Myers and Fine). (3) Inanition results in an increased formation of creatine, accompanied by an inability of the organism to utilize or destroy the surplus creatine (Mendel and Rose; Benedict and Osterberg). The second and third hypotheses have the greatest number of adherents.

The work of Myers and Fine (3) is the most representative and presents the strongest argument for the proponents of the second idea. These workers have shown a preliminary increase in creatine concentration of muscle during early fasting in rabbits, which is followed by a marked decrease as a result of prolonged fasting. They concluded that the resulting creatinuria of fasting is directly related to the decrease in the creatine content of muscle. The chief objections to this work are the suppositions "that creatine may not disappear in anabolic reactions, and that in the starving organism no creatine is ever produced" (1). Their results differ from those of Mendel and Rose (2, *a*), who found a gradually marked increase in the creatine content of muscle with prolonged fasting. Myers and Fine attempt to explain these discrepancies by making calculations from the "initial creatine." This method of calculation lacks experimental evidence.

The data presented in this paper demonstrate a rise in the creatine content of muscle during fasting which is particularly marked during the terminal stage. As a result of kidney analyses, we must assume that a creatinuria existed. These experiments demonstrate, therefore, that during fasting in the rat, creatine is eliminated in the urine with an accompanying progressive increase in the creatine content of muscle. There is every reason to believe from our data that "creatine is a product of endogenous metabolism, and that an increased formation of creatine occurs when the tissue catabolic processes are accelerated" (2, *b*).

The influence of the adipose tissue in muscle and kidneys on the

creatine changes in these tissues must be considered negligible. The fat content of the entire rat body varies from 5 to 8 per cent of the moist weight. Since the muscle and kidneys contain but relatively small amounts of fat, a complete removal of fat at the terminal stage of fasting would account for only a small part of the increase in creatine content noted. It is true that the loss of fat may affect the apparent rise in creatine concentration more markedly during the earlier stages of fasting, but even this influence cannot be considerable in the light of the increases in creatine concentration found.

TABLE XII.

*Showing "Saturation Point" for Creatine Storage in Muscle, together with Data for Other Tissues, under Various Experimental Conditions.*

Figures expressed in per cent.

	Control.	10 per cent creatine diet, fed 1 day.	Refeeding on control diet after extreme inanition.	Extreme inanition.	10 per cent creatine diet, refed after extreme inanition.
Muscle.....	0.454	0.533	0.554	0.570	0.592
Liver.....	0.041	0.266	0.057	0.044	0.432
Brain.....	0.154	0.165	0.145	0.163	0.168
Heart.....	0.217	0.262	0.252	0.230	0.303
Testes.....	0.299	0.290	0.308	0.308	0.313
Kidneys.....	0.055	0.221	0.085	0.083	0.254
Total solids.....	25.1	24.3	23.5	24.3	24.0

Further support for the idea that the loss of fat is unimportant so far as it influences the creatine content of muscle and kidneys is given in Table IV. After a control diet is refed to rats subjected to prolonged fasting, the creatine content of the kidneys returns to normal after 2 days, despite the fact that at this time the rats were 23.2 per cent below the normal weight. In the muscle, the creatine content is rapidly approaching normal by the 4th day of refeeding, though there is still a 17.9 per cent loss of body weight. This rapid decrease in the creatine content of the muscle cannot be explained even by assuming a marked deposition of fat.

Data are summarized in Table XII to illustrate the extreme variations in the creatine concentration of tissues under different

experimental conditions, and particularly in the case of muscle. An increase of 17.4 per cent in the creatine content of muscle is noted after the feeding of a 10 per cent creatine diet to a control animal for 1 day. Since further feeding of creatine is accompanied by a drop in the creatine content of muscle (Table V), we feel this is the highest concentration that can be attained under these circumstances. A still greater increase is obtained as a result of extreme inanition. It is difficult to explain the data obtained for fasting unless it is assumed that creatine is being formed and stored in progressively larger quantities as the rate of catabolism increases. In addition, fasting is unquestionably responsible for the increased creatine capacity of muscle tissue. This view is confirmed by the results obtained in the animals refed a creatine diet after prolonged fasting. In one such animal, for example, the increase amounted to about 45 per cent above the control creatine content of muscle. It seems obvious that the differences in the creatine concentration of muscle caused by feeding creatine to control and fasted animals demonstrates the effect of the nutritional state upon the ability of the muscle to store creatine.

If the assumptions concerning creatine storage are correct, we believe that some fundamental interpretations concerning creatine metabolism may be made. Our evidence would lead one to believe that the storage of excess creatine in tissues is accomplished only under unusual metabolic conditions. Apparently the metabolic state determines the equilibrium between the creatine stored in the tissues and the creatine of the body fluids. It seems that theories attempting to explain creatinuria on the basis of the capacity to store creatine, or because of a "low saturation" point, cannot be considered entirely valid as a result of the evidence presented in this investigation. It is more probable that poor utilization of creatine is responsible for most types of creatinuria.

After feeding diets containing 2.6 per cent creatine for a comparatively long time, Chanutin (9) found no increase in the muscle creatine of rats. In mice, however, a definite increase in muscle creatine content has been demonstrated after a 2 per cent creatine diet is fed (10). In the present experiments, we have been able to demonstrate a marked storage of excess creatine in rat muscle by feeding 5 and 10 per cent creatine diets. It is interesting to point out that the average increase in the muscle creatine of mice



was 9.8 per cent above normal as contrasted with a 20 per cent increase in rats under similar conditions. We must conclude from this evidence that the manner of metabolizing creatine in mice and rats is quantitatively different.

The evidence given thus far emphasizes the importance of the muscle in creatine metabolism. The muscle is the tissue in which the creatine concentration is affected after both fasting and creatine ingestion. The storage of creatine in the liver occurs only after the feeding of creatine. Furthermore, the ability of this organ to store variable amounts of creatine is striking. One can, it is true, ascribe the differences in liver creatine content in these experiments to the rate of absorption from the intestine. After the creatine diet is discontinued, the liver creatine content returns to normal rapidly, while the muscle creatine concentration changes slowly. It appears, therefore, that excess creatine is loosely bound in the liver as compared to the creatine combination in muscle. In previous investigations (9, 10) an attempt was made to correlate creatine metabolism with the liver. In the light of the present studies we must agree with Hunter that the relationship of the liver to creatine metabolism is uncertain, and that "the weight of the evidence on the negative side of the question is greater."

The heart muscle is capable of storing an appreciable amount of creatine. This increase cannot be considered important from a quantitative view-point. It is interesting, however, to note that cardiac tissue is capable of reacting toward creatine in the same manner as striated muscle. The changes in the testes and brain are negligible throughout these experiments. It is doubtful whether the testes and brain with their relatively high creatine content have any relationship to creatine metabolism. It seems that our evidence adds further proof that striated muscle is by far the most important tissue involved in creatine metabolism.

#### CONCLUSIONS AND SUMMARY.

Fasting in the rat causes the following changes in the creatine content and total solids of tissues.

1. The creatine content of rat muscle is progressively increased during the period of fasting. The possible relationship between endogenous catabolic processes and the formation and storage of excess creatine has been pointed out.

2. The kidney creatine content is increased early in fasting, which is certainly indicative of creatinuria.

3. The heart, testes, liver, and brain show very little change in creatine content.

4. Total solids of muscle are increased in the early part of fasting, but decreased at the close of fasting.

The creatine concentration of muscle is increased after feeding a high creatine diet to adult and young rats. The liver and heart also show appreciable increases.

The flexibility of the creatine reservoirs in muscle and liver has been demonstrated. It has been possible to change the saturation point by varying the experimental conditions.

Studies of the blood during and after fasting and during creatine feeding have been made.

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## OXIDATION OF LIGNOCERIC ACID.

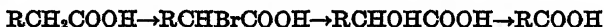
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Recently<sup>1</sup> in the oxidation of a sample of cerebronic acid to the next lower unsubstituted fatty acid, considerable difficulty was experienced in obtaining a pure substance. Instead of pure lignoceric acid, a product was isolated that appeared to be a mixture of acids. It became of interest then, to determine whether an  $\alpha$ -hydroxy acid of known purity related structurally to cerebronic acid could be degraded by oxidation, under conditions similar to those applied to cerebronic acid, to the next lower acid of the series in good yield and uncontaminated by substances that could not be separated easily.

Lignoceric acid was therefore converted into its next lower homologue by passing it through the following steps:<sup>2</sup>



These reactions had been partly carried out by Meyer, Brod, and Soyka<sup>3</sup> in 1913 in their preparation of the docosanoic acid related to lignoceric acid and by Levene and Taylor<sup>4</sup> in 1922. Since the work of Levene and Taylor, it has become known<sup>5</sup> that some higher aliphatic substances are changed on standing so that it is very difficult to bring them back to their original melting points. The materials used by Levene and Taylor having stood for some time, it was desirable to check their figures.

<sup>1</sup> Unpublished experiments.

<sup>2</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913-14, xvi, 475.

<sup>3</sup> Meyer, H., Brod, L., and Soyka, W., *Monatsh. Chem.*, 1913, xxxiv, 1113.

<sup>4</sup> Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1922, lii, 227.

<sup>5</sup> Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1924, lix, 905.

The present experiments show that the melting points recorded both by Meyer, Brod, and Soyka and by Levene and Taylor should be revised upward. A comparison is given, in Table I, of the various determinations.

It is also quite evident that the oxidation of  $\alpha$ -hydroxylignoceric acid gives rise to a single substance, isotricosanoic acid, since extended attempts at purification failed to bring about a significant change in properties. The melting point of the crude acid was 75–77° while the best specimens, obtained by allowing very small fractions to separate from ether, melted at 76.5–77.5°.

The melting point of the isotricosanoic acid brings out an interesting relationship in the lignoceric series. Whereas in the normal acids the curve of melting points of the acids with odd numbers of carbon atoms is considerably below that of the even numbered

TABLE I.

	Meyer, Brod, and Soyka.	Levene and Taylor.	Taylor and Levene.
$\alpha$ -Bromolignoceric acid.....	68–69°	68.5°	69.5–70.5°
$\alpha$ -Hydroxylignoceric acid.....	91–92°	91–92°	94–95°
Isotricosanoic acid.....		73.5°	76.5–77.5°

acids, the curves in the lignoceric series are apparently almost superimposed. In the neighborhood of  $C_{23}$ – $C_{24}$ , in the normal series<sup>4</sup> the odd numbered acids melt at a temperature approximately 1° lower than the preceding even numbered acids. Meyer, Brod, and Soyka<sup>5</sup> prepared the docosanoic acid from lignoceric acid. It melted at 75°. In this series then, the odd numbered acid melts at a point between the melting points of the two adjacent even numbered acids. This constitutes further evidence in favor of the view first advanced by Meyer, Brod, and Soyka, that lignoceric acid is not a normal acid.

Recently Klenk<sup>6</sup> has obtained from cerebronic acid, by oxidation with permanganate, a tricosanoic acid which melts at 78.5°. The relationship of that acid to the acid described here may prove of interest.

<sup>6</sup> Klenk, E., *Z. physiol. Chem.*, 1928, clxxiv, 214.

## EXPERIMENTAL.

The lignoceric acid was prepared from peanut oil by a method that was essentially that described by Levene, Taylor, and Haller.<sup>7</sup> The mixture of insoluble fatty acids from peanut oil was cooled to 20–22° overnight in centrifuge bottles and centrifuged until there was very little further packing of the sediment. The sediment was then washed repeatedly with 95 per cent alcohol, in the centrifuge bottles, until the supernatant solution was nearly colorless and the solid acids had been reduced in volume to about 10 per cent of the original. Further removal of liquid acids was accomplished by three crystallizations from 95 per cent alcohol at 20–24°. The melting point was then 72.5–73.5° and the yield was about 2.5 per cent of the insoluble fatty acids of the peanut oil. Alcohol then failed to raise the melting point. The acid crystallized in lustrous plates.

For final purification the material was crystallized from pyridine and from ether until the melting point was 80–81° when the bath was so heated, with stirring, that the temperature rose 1° in 7 to 8 seconds. It solidified sharply at 77°.

0.1001 gm. substance: 0.2871 gm. CO<sub>2</sub> and 0.1159 gm. H<sub>2</sub>O.

0.5001 " " required 6.74 ml. 0.2 N NaOH.

C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>. Calculated. C 78.26, H 13.04, mol. wt. 368.

Found. " 78.21, " 12.96, " " 371.

This molecular weight, and all others, were obtained by dissolving the acid in a mixture of 50 ml. of methyl alcohol and 25 ml. of toluene and titrating the hot solution with approximately 0.2 N NaOH in the presence of phenolphthalein. A correction of 0.07 ml., the difference between the alkali required to titrate a standard acid potassium phthalate solution in water and in the mixture of methyl alcohol and toluene respectively, was subtracted. The titrations were carried out with a burette graduated in 0.02 ml. divisions. In this way molecular weights are obtained which, with fatty acids of known purity, are uniformly 2 to 3 units higher than the calculated values.

*α-Bromolignoceric Acid.*—Lignoceric acid (3.1 gm.) was melted on the water bath with red phosphorus (0.2 gm.) and bromine

<sup>7</sup> Levene, P. A., Taylor, F. A., and Haller, H. L., *J. Biol. Chem.*, 1924, lxi, 157.

(4.9 gm.) dropped in slowly. The heating was continued for 4 hours, at which time an excess of bromine was still present. The acid bromide was poured into water and stirred until the acid was liberated. It was then collected in ether, dried, and the ether distilled. The residue was crystallized from petroleum ether at 0°. M. p. 67.5–68.5°. The yield was 90 per cent of the theoretical. Two further crystallizations brought the melting point to 69.5–70.5° after which it could be changed no further. Meyer, Brod, and Soyka<sup>3</sup> as well as Levene and Taylor<sup>4</sup> found a melting point of 68–69°.

0.1859 gm. substance: 0.0763 gm. AgBr (Carius).

0.5012 " " required 5.61 ml. 0.2 N NaOH.

$C_{24}H_{47}O_2Br$ . Calculated. Br 17.88, mol. wt. 447.

Found. " 17.47, " " 447.

*$\alpha$ -Hydroxylignoceric Acid.*—The  $\alpha$ -bromolignoceric acid was heated on the water bath with a large excess of 10 per cent sodium hydroxide solution for 40 hours. The cooled solution was neutralized with hydrochloric acid, the soap filtered off, and the acid liberated in the presence of ether with hydrochloric acid. The ether solution was washed with water, dried, and the ether distilled. The residue was crystallized from acetone at 0° and then melted at 93–94°. The yield was 90 per cent of the theoretical. Meyer, Brod, and Soyka<sup>3</sup> and Levene and Taylor<sup>4</sup> record a melting point of 91–92°. The molecular weight was 388. For further purification the acid was converted into the ethyl ester and crystallized from absolute alcohol. M. p. 57.5–59°. The ester was saponified and the acid liberated as before. It now melted at 94–95°. Continued crystallization and passage over the lead salt failed to alter it further.

0.1001 gm. substance: 0.2762 gm.  $CO_2$  and 0.1131 gm.  $H_2O$ .

0.4999 " " required 6.46 ml. 0.2 N NaOH.

$C_{24}H_{48}O_2$ . Calculated. C 75.00, H 12.50, mol. wt. 384.

Found. " 75.24, " 12.65, " " 387.

*Isotricosanoic Acid.*—The  $\alpha$ -hydroxylignoceric acid was dissolved in boiling acetone and treated with a slight excess of potassium permanganate. The solution was then boiled until the purple color was destroyed. After cooling, the mixture of manganese

dioxide and soap was filtered off, dried, and decomposed with sodium bisulfite and hydrochloric acid, the fatty acid being collected in ether. The ether solution was dried and evaporated. The residue crystallized from acetone at  $0^{\circ}$  in lustrous plates and melted at  $75-77^{\circ}$ . The molecular weight was 357 and the yield was 80 per cent of that calculated.

The ethyl ester was prepared in the usual manner and crystallized at  $0^{\circ}$ . It was recrystallized from alcohol and then melted at  $51.2-52.2^{\circ}$ . After distillation at 0.1 mm. it was saponified and the acid liberated in the presence of ether as before. After crystallization from acetone, the melting point was  $76.5-77.5^{\circ}$ . It solidified at  $74.5-74^{\circ}$ . Passage over the lead salt and crystallization from ether failed to change the melting point. It was finally separated into six fractions by allowing its ether solution to evaporate slowly at  $0^{\circ}$  and decanting the supernatant solution after each successive small amount of acid had separated. The melting points of these fractions were identical with the last given above. Levene and Taylor<sup>4</sup> record a melting point of  $73.5^{\circ}$ .

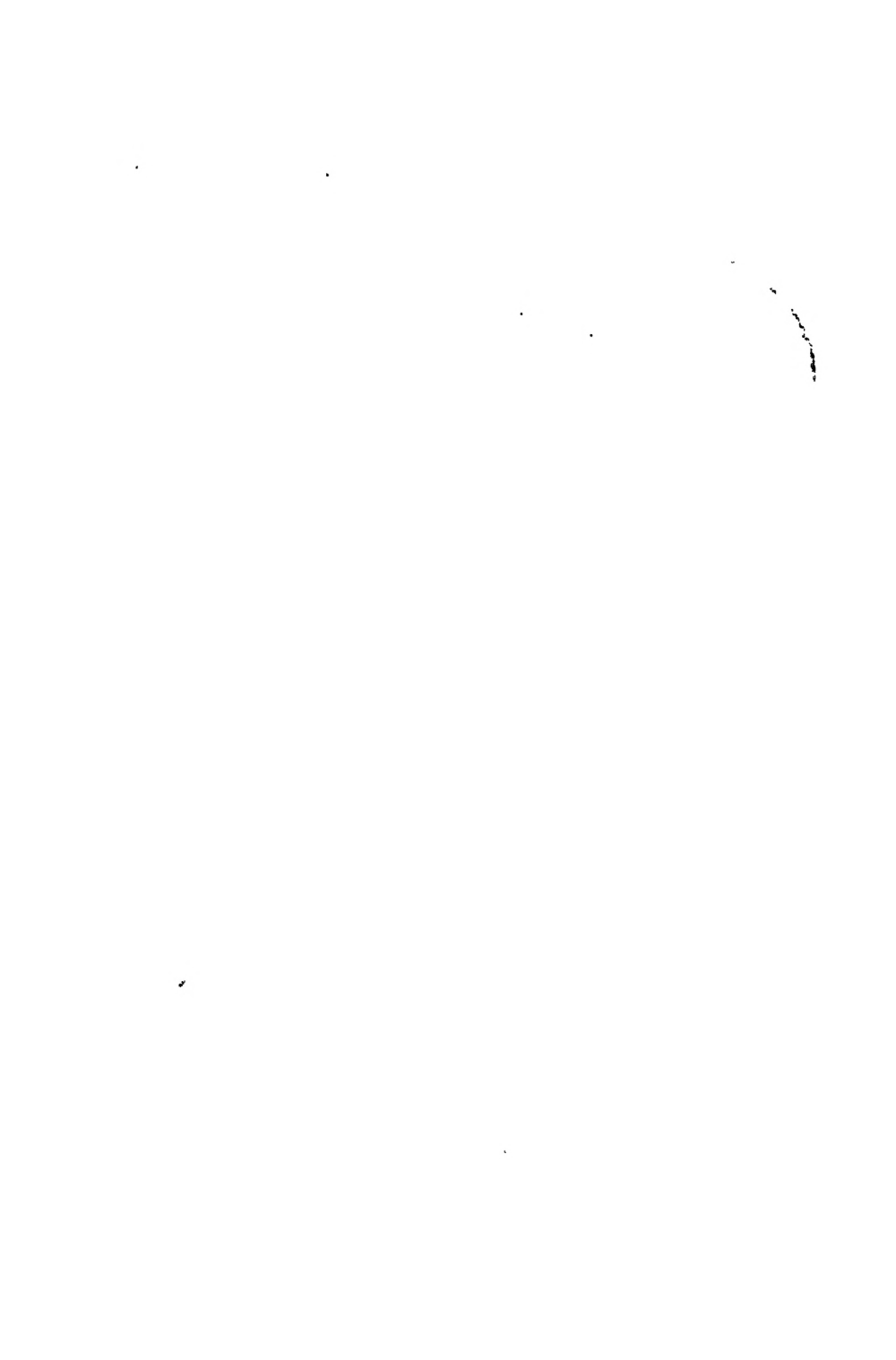
0.1002 gm. substance: 0.2870 gm.  $\text{CO}_2$  and 0.1157 gm.  $\text{H}_2\text{O}$ .

0.5005 " " required 6.99 ml. 0.2 N NaOH.

$\text{C}_{23}\text{H}_{46}\text{O}_2$ . Calculated. C 77.97, H 12.99, mol. wt. 354.

Found. " 78.10, " 12.93, " " 358.

The melting points are corrected.





## THE PHYSIOLOGY OF ERGOTHIONEINE.

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(Received for publication, October 6, 1928.)

The discovery of the presence of ergothioneine in blood by Hunter and Eagles (1) and Benedict and his coworkers (2) has raised the question of its significance in animal physiology. A study has therefore been made of certain factors which have been found to influence the appearance of this substance in the blood of the pig.

As a means of estimating the amount of ergothioneine in blood, use was made of a colorimetric method recently described by Hunter (3) which is a special modification of the well known Ehrlich (4) diazo reaction. This reaction is remarkably specific for the thiolimidazole ring and has been shown by Hunter to yield strictly quantitative results when applied to pure aqueous solutions of ergothioneine. We encountered difficulty, however, when we attempted to recover quantitatively ergothioneine added to bloods or to solutions of purified proteins. An appreciable amount of ergothioneine is apparently adsorbed upon the precipitates produced during the removal of protein by any of the common methods. This finding is analogous to that of Cary and Harding (5) who were unable to recover completely cystine added to blood. The analytical difficulty does not affect the general observations to be recorded in this paper but it does indicate, however, that further studies of methods of protein removal are needed before the "rest N" of the blood may be completely characterized.<sup>1</sup>

Of the various animal bloods so far examined that of pigs ap-

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<sup>1</sup> There are numerous papers in the French literature of the last few years dealing with this subject (6).

TABLE I.  
*Ergothioneine Content of Pig Blood Obtained from Animals Raised under Different Conditions.*

Breed.	Sex.	Age (approximate).	Results of test.		Herd.	Diet.	Remarks.
			Immediate color.	24 hr. ppt.*			
Duroc-Jersey.	M.	6 mos.	—	—	Springside Farm.	Garbage.	
	F.	6 wks.	—	—	"	"	
	"	2-3 yrs.	±	±	"	"	
	"	2-3	—	—	"	"	Nursing young.
	"	M.	3	—	"	"	"
Berkshire.	"	3	—	—	"	"	
	F.	2	22	+	Conn. Agric. Coll.	Corn, wheat middlings, milk.	
Duroc-Jersey.	"	2	15	+	"	"	
	M.	1-2	12	+	"	"	
	"	2	10	+	"	"	
	"	3	4	+	"	"	
Berkshire.	"	3	4	+	"	"	
	F.	3	4	+	"	"	
Not recorded.	Not known.	1-2	30	+	Abattoir (shipped from Iowa).	Grains and grasses.	For only 2 wks. Preceding year on garbage.
	"	1-2	28	+	"	"	

Not re- corded.	Not known.	1-2	27	+	Abattoir (shipped from Iowa).	Grains and grasses.
"	"	1-2	25	+	"	"
"	"	1-2	23	+	"	"
"	"	1-2	22	+	"	"
"	"	1-2	20	+	"	"
"	"	1-2	19	+	"	"
"	"	1-2	18	+	"	"
"	"	1-2	8	+	"	"

\* "In certain blood-filtrates in which the concentration of ergothioneine is very low, or the carnosine content is high, a characteristic color may not develop. Whether the test is positive or negative may however be decided by allowing the mixture to stand over night, when the formation of even a very slight purplish precipitate indicates the presence of ergothioneine. This precipitate appears to form in greater dilutions of ergothioneine in blood-filtrates than in corresponding simple solutions of ergothioneine, so that for qualitative purposes this aspect of the test is of importance." (Hunter (3))

pears to contain ergothioneine in the largest proportion and this material has therefore been used in our experiment. A large number of samples of pig blood obtained from abattoirs in Toronto, New Haven, and Philadelphia have been examined during a period of over 2 years. All of these contained appreciable amounts of ergothioneine. On the other hand, no ergothioneine could be demonstrated in the blood from pigs raised at Springside Farm, New Haven. The most obvious difference between the pigs observed at the abattoir and those at Springside Farm lay in their dietary history. The former had been fattened on grains and grasses whereas the latter had been fed exclusively upon city garbage. It therefore seemed possible that the explanation of our results might be found in this difference in diet. Accordingly samples of blood from pigs of known dietary history, which were obtained through the courtesy of Professor H. L. Garrigus, Connecticut Agricultural College, Storrs, were examined.

A survey of the data summarized in Table I seems to warrant the following deductions:

1. Diet has a significant effect upon the occurrence of ergothioneine in blood.
2. Sex and breed have little or nothing to do with the occurrence of ergothioneine in blood.
3. Age is an influencing factor only when it limits the period during which the animal has been on a dietary régime rich in ergothioneine-producing material.

These results suggest either that some constituent of the diet of the abattoir pigs contains preformed ergothioneine which finds its way into the red blood cells, or that a precursor is present which is converted in the body into ergothioneine. To test this point two Duroc-Jersey pigs of the same litter, 6 weeks old, were obtained from Springside Farm. The bloods of their mother and litter mates showed no evidence of the presence of ergothioneine. Both had been inoculated against *Streptococcus hæmolyticus* 2 days before the first blood sample was taken. One, a female, was fed for 1 month upon a synthetic diet of casein, sucrose, lard, butter, and salts (the Casein III diet of Cowgill (7)). The male was kept at the farm on the regular garbage diet. Water was supplied *ad libitum*. At the end of the period ergothioneine could not be demonstrated in the blood of either animal. Both animals were

TABLE II.  
*Effect of Various Dietary Régimes upon Ergothioneine Content of Pig Blood.*

Date.	Weight.	Results of test.		Diet.
		Imme- diate color.	24 hr. ppt.	
Male pig (castrated 4 wks. before beginning of experiment).				
	<i>kg.</i>			
May 3		—	—	Garbage.
" 8		—	—	"
" 15		±	—	"
" 21	19.7	—	—	" (changed to Corn Diet I after taking blood sample).
" 29	19.4	±	±	Corn Diet I.
June 3	20.0	—	—	" " "
" 9	21.0	—	—	" " "
" 16	22.4	+	+	" " " (changed to Corn Diet II after taking blood sample).
" 23	23.7	++	+	Corn Diet II.
July 1	25.2	++	++	" " "
" 8	27.0	++	++	" " "
" 15	30.0	++	++	" " "
" 21	36.5	++	++	" " "
" 25	38.0	++*	++	" " "
Female pig.				
May 3	13.0	—	—	Garbage (changed to Casein III diet after taking blood sample).
" 8	14.5	—	—	Casein III diet.
" 15	15.3	±	—	" " "
" 21	15.5	—	—	" " "
" 29	17.1	—	—	" " "
June 3	18.6	—	—	" " " (changed to Corn Diet I after taking blood sample).
" 9	21.8	—	—	Corn Diet I.
" 16	23.2	—	—	" " "
" 23	24.8	+	+	" " " (changed to Corn Diet II after taking blood sample).
July 1	25.5	+	±	Corn Diet II.
" 8	27.4	+	±	" " "
" 15	30.0	—	—	" " "
" 21	32.6	±	±	" " "
" 25	35.0	+++	+	" " "

\* Approximately 8 to 9 mg. per 100 cc. of blood on the basis of results obtained in the recovery of known amounts of ergothioneine added to blood samples.

† Approximately 6 mg. per 100 cc. of blood on the basis of results obtained in the recovery of known amounts of ergothioneine added to blood samples.

then placed on Corn Diet I for 20 days and subsequently, owing to the possibility that dietary difficulties might arise from a shortage of vitamins A and D, were transferred to Corn Diet II.

*Corn Diet I.*

Alfalfa meal.....	75 gm.
Cracked yellow corn.....	<i>Ad lib.</i>
Vitavose* (three times a week).....	20 gm.

*Corn Diet II.*

Alfalfa meal.....	75 gm.
Cracked corn.....	<i>Ad lib.</i>
Casein, technical.....	40 gm.
Salt mixture (Osborne and Mendel)†.....	15 "
Yeast†.....	20 "
Cod liver oil.....	5 cc.

\* A wheat germ concentrate obtained from E. R. Squibb and Sons, New York.

† Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

‡ Powdered Yeast Foam Tablets, obtained from Northwestern Yeast Company, Chicago.

The data secured (Table II) while the animals were under observation indicate that ergothioneine or its precursor is present in Corn Diets I and II and is lacking from the Casein III diet of Cowgill as well as from the garbage diet fed at Springside Farm.

The following experiments point to the possibility that the corn diet furnishes a precursor to ergothioneine rather than ergothioneine itself. Thiolhistidine seemed, from many points of view, to be the most likely substance to act as this precursor and, although the substance has not hitherto been detected among the products of hydrolysis of proteins, this consideration led us to apply the Hunter diazo test for the thiolimidazole ring to hydrolysates of the proteins contained in the food mixtures with which the animals were supplied. Samples of purified preparations of these proteins were completely hydrolyzed with sulfuric acid. After exact removal of the sulfate ion sodium hydroxide was added until the solution was faintly acid to litmus. The solutions were then tested for the presence of the thiolimidazole ring, and the results are summarized in Table III.

It is impossible to evaluate in terms of ergothioneine the amount of the reacting compound present in the hydrolysates, because histidine has an interfering action upon the test. Histidine gives a bright yellow color, whereas the thiolimidazole ring (*e.g.*, as in ergothioneine) gives a red color with a purple tinge. Mixtures of

histidine and ergothioneine give a characteristic brownish red color and the depth of red produced is dependent upon the ratio between ergothioneine and histidine in the test sample. The close correspondence between the colors observed when the hydrolysates were tested and those given by certain of the mixtures of histidine and ergothioneine leads us to believe that a substance reacting positively to the Hunter diazo test is present in these hydrolysates.

TABLE III.

*Application of Hunter's Diazo Reaction for Ergothioneine to Protein Hydrolysates.\**

Material hydrolyzed.	Result of test.
Casein.....	-†
Gelatin.....	-
Edestin.....	+
Egg albumin.....	+
Corn, whole.....	+
Zein.....	++
Corn gluten (nearly free from zein).....	+
Wheat gliadin.....	+
“ glutelin.....	+
Alfalfa meal.....	+

\* *Method of the Test.*—Each hydrolysate was so handled that the 2 cc. aliquots used in the test represented 80 mg. of protein. Zein is reported to contain 0.8 per cent histidine and this value was used in making up the standard solutions. Histidine solutions containing in 2 cc. an amount of histidine equivalent to that found in 80 mg. of protein, 0.64 mg., and varying amounts of ergothioneine (0.02, 0.04, 0.06 mg.) were used as standards. The solution containing 0.02 mg. of ergothioneine in 2 cc. gave the most satisfactory color comparison.

† In a negative test only the clear yellow color characteristic of histidine appeared.

Because of the lack of knowledge of the color value of this reacting substance, its quantitative evaluation, in proteins in terms of thiohistidine or ergothioneine, is at present impossible.

It is of interest to note that the casein and gelatin hydrolysates developed only the yellow color characteristic of histidine, indicating the absence of the reactive substance from these proteins. This observation is in agreement with the results of the feeding experiments in which casein was employed.

## SUMMARY.

Evidence obtained from feeding experiments as well as from the application of Hunter's modification of the diazo color reaction to protein hydrolysates indicates that certain proteins yield a substance capable of acting in the living organism as a precursor of the ergothioneine. It is suggested that this precursor may be thiolhistidine. Further study of this problem is now being carried on.

The physiological significance of the presence of ergothioneine in the red blood corpuscles remains unknown. Until further study of the use of protein precipitants is made it cannot be definitely stated that ergothioneine is entirely absent from pig blood under certain definite dietary conditions. It is evident from these experiments, however, that the content of ergothioneine in the blood of pigs can be raised from an undetectable amount to a measurable quantity.

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## COLORIMETRIC DETERMINATION OF INORGANIC SULFATE IN SMALL AMOUNTS OF URINE.

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(Received for publication, October 9, 1928.)

### *Principle.*

The inorganic sulfate is precipitated as benzdine sulfate. The precipitate is diazotized and coupled with phenol in an alkaline medium to produce a yellow color which is proportional to the amount of benzdine. This is compared in a colorimeter with a similarly treated standard sulfate solution.

### *Procedure.*

Filter the urine through a filter paper and place 5 cc. of the filtered urine in a 10 cc. graduated cylinder. Test with litmus paper, and if not acid, acidify with 5 per cent acetic acid. In either case dilute to 10 cc. with distilled water.

Place 2 cc. of the diluted urine in a clean centrifuge tube. Add 2 cc. of benzdine reagent and 4 cc. of 95 per cent alcohol. Mix thoroughly by twirling and allow to stand for 15 minutes.

Centrifuge at moderate speed for 5 minutes and carefully decant the supernatant fluid, touching the lip of the tube against a filter paper to remove the last drop.

Add 5 cc. of 50 per cent alcohol to the precipitate. Add this in small portions and shake the tube between each addition so as to suspend the precipitate completely. Centrifuge and decant the supernatant fluid as before. Repeat this procedure once more.

To the precipitate add 0.5 cc. of concentrated HCl and 5 cc. of distilled water in small portions, shaking between each addition. Add 1 cc. of 10 per cent sodium nitrite solution and allow to stand for 5 minutes.

*Preparation of Standard.*

With an accurate pipette measure 2 cc. of the standard ammonium sulfate solution into a clean centrifuge tube. Add 2 cc. of the benzidine reagent and 4 cc. of 95 per cent alcohol and allow to stand for 15 minutes. Centrifuge, pour off the supernatant fluid, and wash twice with 50 per cent alcohol in the manner previously described. Diazotize the resulting precipitate by adding 0.5 cc. of concentrated HCl, 5 cc. of water, and 1 cc. of 10 per cent sodium nitrite, as before.

At the end of 5 minutes transfer the unknown and the standard to separate 100 cc. cylinders. Rinse the centrifuge tubes twice with 10 cc. portions of water, adding the washings to the respective cylinders.

Add to each cylinder 5 cc. of 15 per cent NaOH and 5 cc. of sodium phenolate. Make up to the 100 cc. marks with water. If the color of the unknown is too deep for accurate comparison in the colorimeter, withdraw an aliquot portion and dilute with water until an approximate match for the standard is obtained. Compare in the colorimeter with the standard set at 20 mm.

*Calculation of Results.*

$$\frac{S}{R} \times 0.1 \times \frac{DR}{DS} \times 100 = \text{mg. sulfur in 100 cc. urine.}$$

$S$  = reading of standard.

$R$  = " " unknown.

$DR$  = dilution of unknown.

$DS$  = " " standard.

*Reagents.*

1. *Benzidine Reagent.*—In a 500 cc. cylinder place 4 gm. of pure benzidine hydrochloride; add 10 cc. of concentrated HCl and distilled water to the 500 cc. mark. Mix well until dissolved and filter. If the solution has a brown tinge, due to impurities in the benzidine hydrochloride, shake the solution with a few gm. of animal charcoal, let stand for a few hours, and filter. A colorless solution will thus be obtained.

2. *Standard Ammonium Sulfate.*—Weigh out accurately 4.1216 gm. of pure dry ammonium sulfate and dissolve in water in a liter volumetric flask and make up with water to the mark. 1 cc. of

this solution contains 1 mg. of sulfur. This is the stock solution. The standard used in the test contains 0.1 mg. of sulfur in 2 cc. It is prepared by diluting the stock solution 1:20. Both the stock solution and the dilute standard are preserved with chloroform.

3. *Sodium Phenolate*.—Dissolve 50 gm. of phenol crystals in about 500 cc. of water and add 15 per cent NaOH until alkaline to litmus paper. Dilute with water to 1 liter.

TABLE I.

Tube No.	P	S	Colorimetric readings compared to Tube 8, set at 20 mm.
	<i>mg.</i>	<i>mg.</i>	<i>mm.</i>
1	2.5	0.1	20.7
2	5.0	0.1	20.2
3	7.5	0.1	20.6
4	10.0	0.1	19.9
5	12.5	0.1	20.3
6	20.0	0.1	9.8
7	25.0	0.1	8.5
8	None.	0.1	20.3

## DISCUSSION.

*Factors Influencing Precipitation of Benzidine Sulfate.*

Phosphates and chlorides in solution interfere with the determination of sulfate by the proposed method unless certain definite conditions obtain at the time of precipitation.

Benzidine phosphate is insoluble and is precipitated along with the sulfate, so that some means must be devised to prevent the precipitation of the phosphates.

Fiske (1) who devised a volumetric method for the determination of sulfate by the use of benzidine, removes the phosphates from solution with magnesia mixture previous to the precipitation of the benzidine sulfate. However, it has been shown by Rosenheim and Drummond (2) that the proper adjustment of the acidity will keep the phosphates in solution, thus preventing their being precipitated by the benzidine. They advise making the urine acid to Congo red before addition of the benzidine solution.

Gauvin and Skarzynski (3) add the same amount of acid to every urine, regardless of the initial acidity, obtaining good results. This was later found to be true by Drummond (4).

The amount of acid used in our benzidine reagent is capable of taking care of phosphates in amounts far in excess of any quantity that may be present in urine in the amount and dilution used by us. Table I shows that the acidity in our benzidine reagent can take care of a great excess of phosphate which is ordinarily present in urine.

TABLE II.  
*Effect of HCl and NaCl.*

Tube No.	Concentrated HCl.	S	Colorimetric readings compared to Tube 10, set at 20 mm.	NaCl (10 per cent).	S	Colorimetric readings compared to Tube 10, set at 20 mm.
	cc.	mg.	mm.	cc.	mg.	mm.
1	0.01	0.1	20.4	0.1	0.1	20.1
2	0.02	0.1	20.2	0.2	0.1	20.0
3	0.03	0.1	20.0	0.3	0.1	20.3
4	0.04	0.1	19.7	0.4	0.1	19.8
5	0.05	0.1	19.9	0.5	0.1	20.2
6	0.08	0.1	20.6	0.6	0.1	20.6
7	0.1	0.1	22.3	0.8	0.1	21.2
8	0.2	0.1	27.8	1.0	0.1	22.6
9	0.3	0.1	44.0	1.5	0.1	25.3
10	None.	0.1	20.1	None.	0.1	19.9

Eight tubes were set up containing 0.1 mg. of sulfur and 0.05 cc. of concentrated HCl each. Varying amounts of phosphate were added to the first seven tubes. The eighth tube contained no phosphate and was used as a control.

Hydrochloric acid and its salts when present above certain concentrations inhibit the precipitation of benzidine sulfate. Fiske (1) and Rosenheim and Drummond (2) guard against an excess of HCl by preliminary removal of the phosphates, thus obviating the necessity for the use of high concentrations of HCl.

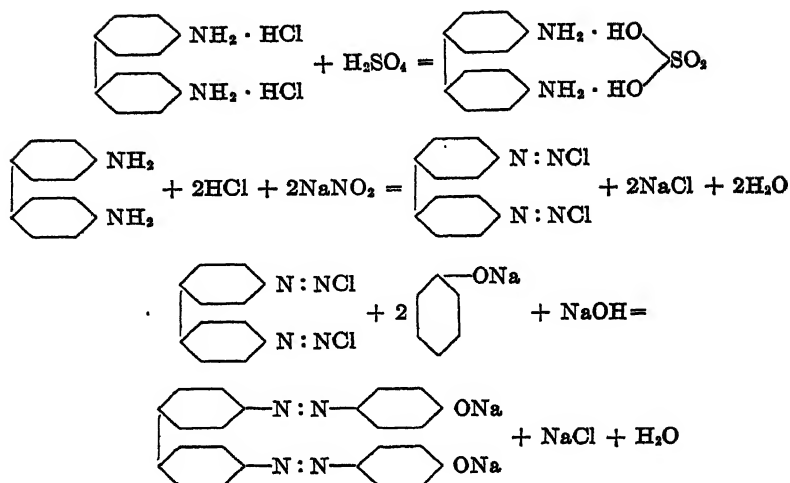
We have studied quantitatively the effect of HCl and of NaCl on the precipitation of benzidine sulfate, and as Table II shows HCl and NaCl do not interfere even when in far greater amounts than can possibly be present at any stage in the procedure.

It is necessary that the urine be slightly acid since in an alkaline medium benzidine base will precipitate. To guard against the slight solubility of benzidine sulfate in aqueous solution the precipitation and washings are carried out in 50 per cent alcohol. It was found that benzidine sulfate is highly insoluble in such a medium. Repeated washings of the benzidine sulfate with 50 per cent alcohol failed to cause any loss of benzidine sulfate.

When the supernatant fluid is decanted at each washing great care must be taken not to lose any of the precipitate since the benzidine sulfate is crystalline and does not pack tightly at the bottom of the tube. For those who find it difficult to decant properly without loss, an alternate procedure of filtration instead of centrifugalization may be used. The sulfate is precipitated with benzidine hydrochloride in an alcoholic medium and after standing for 15 minutes is poured on a small filter paper (Whatman No. 42) and the washings carried out by addition of small amounts of 50 per cent alcohol. When completely washed puncture the tip of the filter paper cone and wash the precipitate with about 20 cc. of water from a wash bottle into a 100 cc. cylinder. From here the procedure is the same as already described.

#### *Color Production.*

The color produced is due to a simple diazo dye. Benzidine is easily diazotized by nitrous acid at room temperature to produce the tetraazo salt. The sulfate radicle in the benzidine sulfate does not interfere since benzidine is a very weak base and its sulfate readily dissociates. The tetraazo salt is capable of being coupled in an alkaline medium with a vast series of phenol or naphthol derivatives, either singly or in varied combinations. The variety of colors that can be produced in this manner is very great. Not all the dyes thus produced can be adapted to colorimetric determinations as some are highly insoluble and some are of such great tinctorial power as to necessitate unwieldy dilutions. After many of the possible combinations had been tried, phenol was found to be the ideal reagent for color development. The phenol dye formed is soluble in aqueous solutions, is highly stable, and its tinctorial power does not demand excessive dilutions. The following reactions take place:



The amount of alkali within certain broad limits does not influence the development of the color; thus when a number of determinations were made with 15 per cent NaOH varying between 2.5 and 20 cc., the color developed faster in the tubes containing

TABLE III.

Tube No.	S present in 100 cc. urine.	S added to 100 cc. urine.	S found.	Theoretical values.
	mg.	mg.	mg.	mg.
1	33.0	10	44.2	43
2	33.0	20	52.3	53
3	33.0	30	65.1	63
4	33.0	50	81.4	83
5	33.0	70	101.8	103

less alkali, while the larger the amount of alkali the longer it took for the color to develop. On standing for some time the same amount of color was developed in all the tubes. Thus an excess of alkali retards the color development.

An excess of phenol has no material effect upon the development of the color.

The addition of known amounts of sulfur (in the form of sulfate) to urine showed good recoveries, as is shown in Table III.

The method was also checked against gravimetric determinations. The results are recorded in Table IV.

TABLE IV.

Tube No.	S in 100 cc. urine.	
	Colorimetric method.	Gravimetric method.
	<i>mg.</i>	<i>mg.</i>
1	54.6	53.0
2	15.3	14.8
3	34.1	33.4
4	107.0	103.2
5	38.5	37.2
6	18.9	18.4
7	41.8	41.3
8	45.4	44.5
9	61.2	60.7

## SUMMARY.

A colorimetric method is described for the determination of inorganic sulfate in small amounts of urine. The sulfate is isolated as benzidine sulfate. The benzidine is diazotized and the color developed with phenol in an alkaline solution.

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# BLOOD SUGAR DETERMINATION AND SEPARATION OF SUGARS WITH LIVE YEAST.

A CORRECTION.

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(Received for publication, October 22, 1928.)

In connection with the paper bearing the above title,<sup>1</sup> Dr. Stanley R. Benedict has called our attention to his discussion, which we had overlooked, of Somogyi's conclusion that some sugars may be removed from dilute solution through adsorption by living yeast cells.<sup>2</sup> In view of the evidence which Benedict has presented, favoring the view that the process of removal of sugars from dilute solution by live yeast is solely one of fermentation, it seems desirable to substitute the term "disappearance" for "adsorption" as used in our paper.

Our interest lay in the rapid and selective nature of the process and we made no attempt to investigate its mechanism or to distinguish between adsorption, diffusion, fermentation, *etc.*

Attention should be called to the importance of avoiding too low a temperature in employing the method which we described, 18–20° being probably the safe lower limit. Temperatures much below this, Benedict has shown, considerably reduce the rate of disappearance of glucose.

<sup>1</sup> Raymond, A. L., and Blanco, J. G., *J. Biol. Chem.*, 1928, lxxix, 649.

<sup>2</sup> Benedict, S. R., *J. Biol. Chem.*, 1928, lxxvi, 464.



## HEXOSEDIPHOSPHATE.

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New York.)

(Received for publication, October 19, 1928.)

The problem of the structure of the hexosediphosphate which forms during enzyme fermentations has attracted the attention of many investigators. Lebedev<sup>1</sup> has shown that the osazone, in whose formation one phosphate has been eliminated, gives glucosazone on alkaline hydrolysis; Meyerhof and Lohmann,<sup>2</sup> using Willstätter's iodate method, have found that the diphosphate is almost pure ketose. These two observations have been corroborated by the isolation of fructose after both acid<sup>3</sup> and enzyme<sup>4</sup> hydrolysis of the diphosphate. Therefore although Kluyver and Struyk<sup>5</sup> do not agree, it seems most probable that the diphosphate is a derivative of fructose.

It has been found, moreover, that on preparing the hydrazone of the diphosphate, both phosphate groups are retained, while on forming the osazone one is eliminated.<sup>6</sup> This has been interpreted as indicating that the labile phosphate is attached to carbon atom (1). The argument receives support from work of our own (unpublished) in which it was found that synthetic 1-fructose phosphate, prepared from  $\beta$ -diacetone fructose, behaves identically. Here also the phosphate is retained in the hydrazone and eliminated with the formation of glucosazone.

These results lead to the conclusion that the ester is fructose

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<sup>1</sup> Lebedev, A., *Biochem. Z.*, 1910, xxviii, 213.

<sup>2</sup> Meyerhof, O., and Lohmann, K., *Naturwissenschaften*, 1926, xiv, 1277.

<sup>3</sup> Young, W. J., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 528.

<sup>4</sup> Harden, A., and Young, W. J., *Proc. Roy. Soc. London, Series B*, 1910, lxxxii, 321.

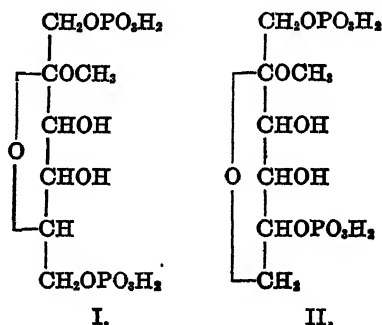
<sup>5</sup> Kluyver, A. J., and Struyk, A. P., *Naturwissenschaften*, 1926, xiv, 882.

<sup>6</sup> Lebedev, A., *Biochem. Z.*, 1910, xxviii, 213; 1911, xxxvi, 243. Young, W. J., *Biochem. Z.*, 1911, xxxii, 178.

diphosphate with one phosphate attached to the first carbon atom. With regard to the second phosphate, however, no evidence has been presented and the present paper deals briefly with this phase of the problem.

As has been shown, the glucosides exhibit a quite different behavior on acid hydrolysis depending upon their belonging to the  $<1, 4>$  or  $<1, 5>$  ring form. Similar effects have been found in the case of the fructosides and we thus appear to have a general method of distinguishing the five-membered from the six-membered lactal glucosides.

In the case of the hexosediphosphate glucoside a  $<2, 5>$  ring would be required if the second phosphoric acid radical were attached to carbon atom (6) (Formula I), and would be prevented if it were on carbon atom (5) (Formula II), while the other possible positions of the phosphate would permit either ring form.



Thus by preparing the diphosphate glucoside and subjecting it to hydrolysis with dilute acid we might hope to obtain valuable information as to the allocation of the second phosphate.

Actually the hydrolysis has been done by Morgan<sup>7</sup> in his excellent paper on the diphosphate glucosides but he apparently did not consider its significance and the conditions which he chose were not such as to make the results conclusive as to the lactal structure. It is possible to conclude from his data, however, that the  $\alpha$ - and  $\beta$ -glucosides have the same lactal ring as their rates of hydrolysis are of the same order of magnitude. We have repeated the preparation of the glucoside, modifying Morgan's

<sup>7</sup> Morgan, W. T. J., *Biochem. J.*, 1927, xxi, 675.

procedure slightly, and have subjected it to hydrolysis with 0.1 N hydrochloric acid in a steam bath. Under these conditions the  $\gamma$ -glucosides are very rapidly hydrolyzed while normal derivatives show only slight hydrolysis.

In the case of the diphosphate it was found that the reduction increased very rapidly at first (Fig. 1) and then quite slowly for

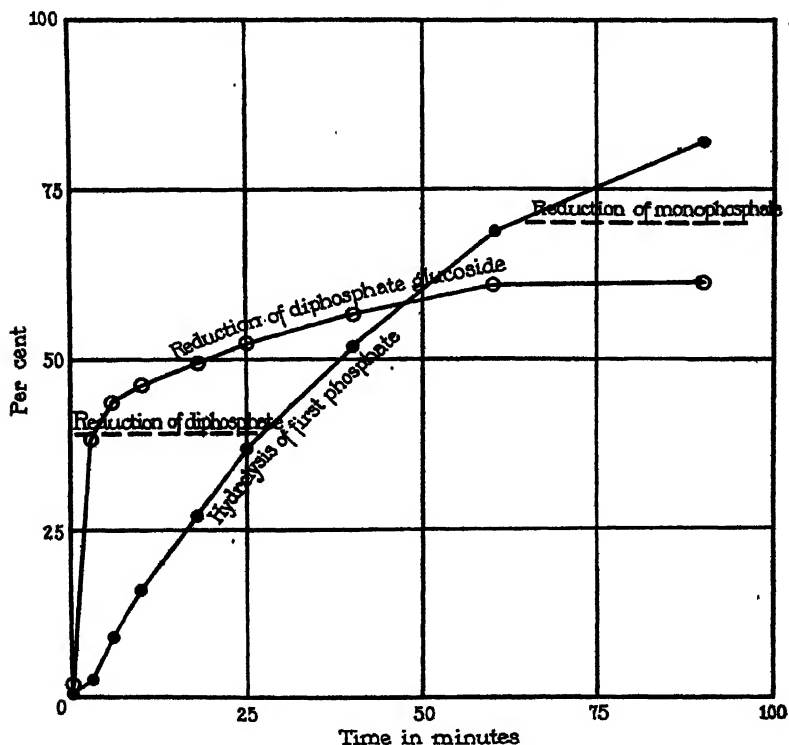
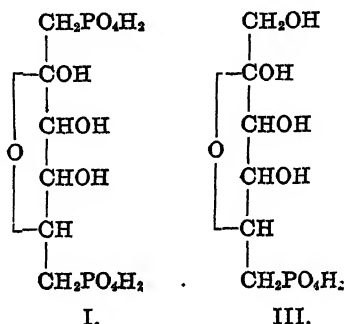


FIG. 1.

the duration of the experiment (90 minutes). The latter slow increase was apparently due to the hydrolysis of the diphosphate to monophosphate, as was shown by the increase in inorganic phosphate, while the first rapid increase represented the hydrolysis of the glucoside to form the diphosphate. This view was confirmed by the fact that the reduction of the diphosphate cor-

responded to the first change as is seen in the figure, while the final reduction value approached that of the monophosphate as a limit.

The very rapid hydrolysis of the glucoside makes it reasonably certain that we are dealing with a  $\gamma$  derivative and this in turn, from what has been said before, leads to the conclusion that the second stable phosphate is on carbon atom (6). The hexosediphosphate therefore has the structure (I) while the Neuberg ester must have the corresponding structure (III).



#### EXPERIMENTAL.

1. *Preparation of Hexosediphosphate Glucoside*.—Candiolin<sup>6</sup> was converted to the sodium salt and then to the barium salt and the latter was very thoroughly dried. 100 gm. of the dry salt were dissolved in 2 liters of dry methyl alcohol containing 34 gm. of dry hydrogen chloride gas as described by Morgan.<sup>7</sup> After standing 24 hours at room temperature the solution was neutralized by passing in dry ammonia gas until faintly alkaline to moist litmus paper. The barium salt which precipitated was filtered with suction, washed with methyl alcohol, and dried *in vacuo*. The dry salt was powdered and repeatedly extracted with water. The aqueous extracts were concentrated under reduced pressure and the pH adjusted to 9.0. An equal volume of 95 per cent alcohol was added with stirring and the salt thus precipitated was purified by repeated solution and precipitation.

<sup>6</sup> We wish again to thank the Winthrop Chemical Company, Inc., for giving us this material.

<i>Analysis.</i>	0.0937 gm. substance: 0.0658 gm. BaSO <sub>4</sub> .
	0.0937 " " : 0.0312 " Mg <sub>3</sub> P <sub>2</sub> O <sub>7</sub> .
	0.1462 " " : 0.0556 " AgI.
C <sub>7</sub> H <sub>12</sub> O <sub>12</sub> P <sub>2</sub> Ba <sub>2</sub> .	Calculated. Ba 43.96, P 9.94, OCH <sub>3</sub> 4.96.
	Found. " 41.32, " 9.27, " 5.02.

2. *Hydrolysis*.—1.0 gm. (dry 0.94 gm.) of the barium salt prepared as above was dissolved in water, the barium was quantitatively precipitated with sulfuric acid, and the mixture was diluted to 21.0 cc., and centrifuged. The solution was filtered to remove a slight turbidity and cooled in ice water. To 18.0 cc. were added 5.2 cc. of ice-cold 0.45 N hydrochloric acid. The final solution contained 0.1 N hydrochloric acid and 0.055 molal ester (equivalent to 10 mg. of hexose per cc.).

Into small Pyrex test-tubes were pipetted 2.5 cc. of the above solution. These were frozen in a solid CO<sub>2</sub>-alcohol bath and were kept frozen in an ice-salt mixture. The tubes were sealed in a blast lamp and replaced in the ice-salt bath. These operations were all performed as rapidly as possible to prevent hydrolysis.

The sealed tubes, with one exception, were removed from the ice-salt and transferred to a boiling water bath. At measured time intervals a tube was removed, shaken well, immediately refrozen in the alcohol-solid CO<sub>2</sub> and replaced in the ice-salt mixture. As soon as possible the tubes were opened and the contents allowed to melt. Samples were then removed for reduction and inorganic phosphate determinations which were done immediately.

3. *Analytical*.—The phosphate determinations were done by the method of Kuttner-Cohen modified as previously described.

For the reduction determinations the Lehmann-Maquet<sup>9</sup> technique was employed but as the material was limited in amount, the method was modified by using only one-tenth as much of each reagent as indicated in the usual procedure.<sup>10</sup> The results were quite as satisfactory for our purpose as when the larger quantities were employed although the accuracy was somewhat less.

The reduction was also determined on solutions of the mono-

<sup>9</sup> Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, 1928, lxxix, 621.

<sup>10</sup> See Griesbach, W., and Strassner, H., *Z. physiol. Chem.*, 1913, lxxxviii, 199.

and diphosphates which were prepared by removing the barium from known quantities of the pure barium salts with sulfuric acid. The monophosphate had been prepared by hydrolysis of the diphosphate and purified by repeated precipitation of the barium salt from aqueous solution with alcohol. The diphosphate had been purified by recrystallizing its strychnine salt and then converting to the barium salt.

In order to standardize the results with the technique employed, the reduction was determined for a pure sample of fructose and the analyses were corrected by the factor thus found. In Fig. 1 the reduction is expressed in per cent and plotted as a function of the time of hydrolysis. In making the calculation the theoretical reduction corresponding to complete hydrolysis to fructose was taken as standard. The reductions of the mono- and diphosphates similarly expressed, are included for comparison. Also in the same figure, there is given the change in inorganic phosphate, expressed as percentage of the first phosphate hydrolyzed at a particular time.

#### SUMMARY.

On the basis of the rate of hydrolysis of the methyl glucoside of the fructose diphosphate of fermentation, it is concluded that the substance has the  $<2, 5>$  lactal structure.

From this it follows that the stable phosphoric acid residue is attached to carbon atom (6) and also that Neuberg's monophosphate is 6-fructose phosphate.



## CLINICAL CALORIMETRY.

### **XLIII. A COMPARISON OF THE THRESHOLDS OF KETOSIS IN DIABETES, EPILEPSY, AND OBESITY. \***

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#### INTRODUCTION.

During the past decade the importance of the formation and the excretion of the acetone bodies has been emphasized. The reports of investigators have varied regarding the ratios of ketogenic-antiketogenic substances which could be metabolized in the body without the production of ketosis. Recently, in studies of obesity, it has been reported that no ketosis developed when the subjects were taking weight-reducing diets.

An attempt has been made in our laboratory during the past year to find the threshold of ketosis for different conditions and to discover reasons for its variation in different subjects.

#### LITERATURE.

No attempt will be made to cover the literature completely. The reader is referred to Shaffer (1) for a review of the earlier work on ketosis. Shaffer (1-4), Woodyatt (5), McCann, Hannon, Perlzweig, and Tompkins (6), and Richardson and Ladd (7) found that the threshold of ketosis was reached when substances having ratios of 1 molecule of ketogenic substance to 1 molecule of antiketogenic were being metabolized in the body. Wilder and Winter (8) and Ladd and Palmer (9) reported the threshold at higher

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\* A summary of the work covered in this paper was presented before the American Society for Clinical Investigation at Washington, D. C., April 30, 1928.

ratios while Wilson, Levine, and Rivkin (10), studying the metabolism of children in a cot chamber, found the threshold at lower ratios than the first group of investigators mentioned. Higgins, Peabody, and Fitz (11) observed one subject who showed very little ketosis while on a carbohydrate-free diet for 3 days. Means (12) and Folin and Denis (13) noted no ketosis in their obese subject, Mrs. McK., except toward the ends of fasting periods of 4 to 5 days in length. Recently Mason (14), in following a number of obese patients on reducing diets who were metabolizing foodstuffs of ketogenic-antiketogenic ratios well above 2:1 (Shaffer), observed no evidence of ketosis as determined by urinary ferric chloride tests.

### *Methods.*

Detailed studies have been made of six subjects in the metabolism ward and in the calorimeter of the Russell Sage Institute of Pathology. Two arctic explorers and one laboratory worker ate nothing but meat, one for 90 days, one for 53 days, and one for 10 days. They received no carbohydrate in their food except that present in the meat in the form of glycogen or its precursor, lactic acid. Their meat diet was essentially a high fat diet for they obtained 75 to 80 per cent of their calories from fat. Also one obese man was observed for 118 days during weight reduction, one epileptic for 84 days, and one diabetic for 99 days. In the obese, epileptic, and the diabetic subjects the carbohydrate in the diet was restricted, so that for considerable periods they were metabolizing foodstuffs of FA:G ratios well above 1.5. All ratios were calculated by Woodyatt's formula:

$$\frac{\text{Fatty acids (FA)}}{\text{Glucose (G)}} = \frac{0.46 \text{ gm. protein} + 0.9 \text{ gm. fat}}{0.58 \text{ gm. protein} + 0.1 \text{ gm. fat} + \text{gm. carbohydrate}}$$

The respiratory metabolism of these men was frequently determined in the calorimeter for periods of 3 to 4 hours both in basal condition and following food. In calculating the foodstuffs metabolized for the period of observation, the maintenance requirement was estimated as the basal metabolism for 24 hours plus an amount which varied, according to the activity of the subjects, from 20 to 75 per cent of their basal requirement. The protein metabolized was estimated by multiplying the urinary nitrogen by 6.25; nitrogen in the feces was ignored. The carbohydrate in the food was considered entirely utilized, and it was assumed that fat accounted for the remainder of the maintenance requirement.

We also calculated the foodstuffs utilized during the calorimeter observations. Here the protein metabolism was determined from the urinary nitrogen and deducted from the total metabolism, leaving the portion due to fat and carbohydrate. Then the proportions and actual amounts of fat and carbohydrate metabolized were determined by using the tables of Zuntz and Schumburg as modified by Lusk (15). From these data the FA:G ratios for the calorimeter periods were obtained.

The ketosis was followed by daily qualitative sodium nitroprusside (Le Nobel) tests of the urine and when they were positive the total acetone bodies were determined quantitatively by the method of Van Slyke (16). When a trace of acetone was detectable by the qualitative test, the quantitative determination would show 0.1 to 0.2 gm. of total acetone bodies calculated as acetone. The threshold of ketosis, defined by Shaffer (4) as the excretion of 0.1 gm. of total acetone bodies in the urine in 24 hours, has been accepted in our laboratory. No effort was made to estimate the amount of acetone eliminated through the lungs.

The accuracy of the calorimeter observations was controlled by frequently interspersed alcohol checks. The detailed results of these checks will be published in our final reports. The average respiratory quotient for the ten checks was 0.663 as compared with the theoretical quotient for alcohol of 0.667.

#### EXPERIMENTAL.

The constancy of the respiratory quotient under the experimental conditions was striking. The quotients were determined for 3 or 4 hour periods with the men in basal condition and also at different times after meals. The ratio between the amounts of protein and fat metabolized remained nearly the same, even when the total metabolism increased 10 to 20 per cent after meals. The men who were taking the meat diets had received no carbohydrate food for at least 7 days before the calorimeter observations and, likewise, the other men had taken and were taking only small amounts of carbohydrate, so that all were metabolizing food materials of FA:G ratios well above 1.5.

Chart I shows the levels of the respiratory quotients for 24 basal observations including all the subjects. Each line represents the average respiratory quotient for 3 consecutive hours.

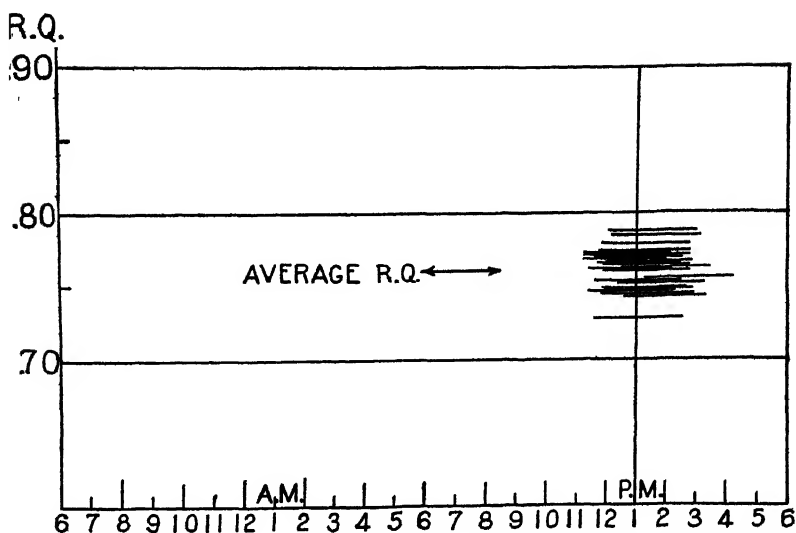


CHART I. Basal respiratory quotients. Each line represents an individual observation extending over 3 hours.

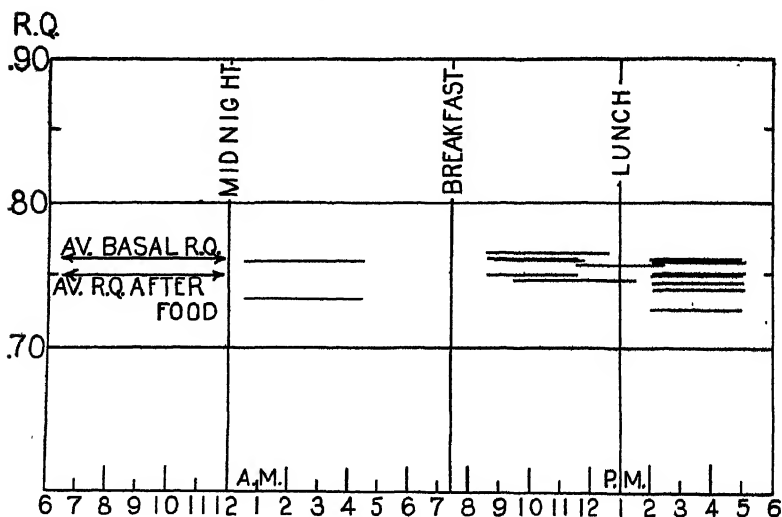


CHART II. Respiratory quotients after food. Each line represents an individual observation of 3 or 4 hours duration.

TABLE I.  
*Relation between FA:G Ratios of Foodstuffs Metabolized and Urinary Acetone  
Body Excretion; Significant Data, by Periods, of Six Subjects  
Metabolizing Foodstuffs with High FA:G Ratios.*

[illegible]

TABLE I—*Concluded.*

Subject, age, weight, and date.	Period No.	No. of days in period.	Average daily N <sub>2</sub> in urine.	Food metabolized, average per 24 hrs.			FA:G, Woodyatt.	Average daily acetone bodies in urine as acetone.	Remarks.
				Protein (N <sub>2</sub> X 6.25).	Fat (by calculation).	Carbohydrate (ingested).			
S. A., 32 yrs., 45 kg. Diabetes. Feb., 1926- May, 1926.	1	7	3.1	19	127	132	0.8	0.12	Av. basal = 1200 cal. 50% of basal = 600 " Total = 1800 " Estimated requirement taken as 1800 cal. Urine was sugar-free for all periods with 25 to 40 units of insulin daily.
	2	3	3.0	19	140	102	1.1	0.07	
	3	4	2.8	18	142	100	1.1	0.11	
	4	7	2.8	18	151	79	1.4	0.34	
	5	8	2.8	18	157	66	1.6	0.53	
	6	13	3.0	19	162	53	1.9	1.03	
	7	4	2.7	17	168	40	2.4	1.91	
	8	6	2.9	18	172	31	2.8	3.70	
	9	6	2.4	15	164	53	2.0	0.92	
	10	5	2.8	18	172	30	2.8	2.96	
	11	6	2.4	15	164	53	2.0	1.00	
	12	5	2.3	14	153	79	1.4	0.12	
	13	6	2.5	16	137	113	1.0	0.11	
	14	4	2.3	14	135	120	0.9	0.21	
	15	3	2.8	18	139	105	1.0	0.49	
	16	6	3.5	22	138	105	1.0	0.27	
R. D., 22 yrs., 60 kg. Epilepsy. Nov., 1927- Jan., 1928.	1	5	9.2	58	149	68	1.4	0.91§	Av. basal = 1264 cal. 50% of basal = 632 " Total = 1896 " Estimated requirement taken as 1900 cal.
	2	8	5.5	34	158	72	1.5	1.06	
	3	14	5.0	31	150	93	1.2	0.10	
	4	3	6.7	42	156	68	1.5	0.45	
	5	7	6.1	38	166	48	1.9	1.68	
	6	3	6.3	39	176	25	2.7	4.93	
	7	19	4.5	28	171	48	2.1	1.42	
	8	3	5.3	33	179	25	2.8	2.05	
	9	6	5.0	31	170	46	2.1	1.61	
	10	9	4.4	27	162	69	1.6	0.56	
	11	7	12.5	78	106	145	0.7	0.0	

\* Average last 7 days of period.

† Estimated.

‡ Average last 2 days of period.

§ Last 2 days only.

All the quotients are between 0.72 and 0.78 and the average is 0.761. In Chart II similar data are presented for sixteen observations following meals, some after a breakfast of lean meat only, or lean and fat meat together, some in the afternoon after breakfast and luncheon, and two during the night after the usual three meals and activity of the day. The average quotient for this group is 0.751.

Table I contains for each individual the findings which relate to the comparison of the FA:G ratio and the acetone bodies excreted

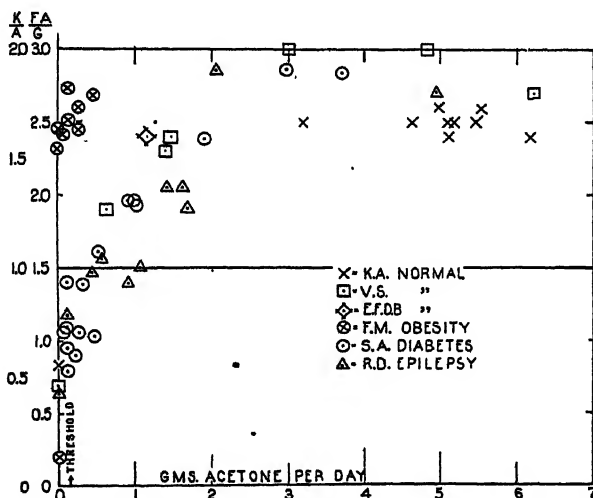


CHART III. Average FA:G ratios of foodstuffs metabolized plotted against average daily urinary acetone excretion for periods of 3 to 30 days.

during given periods. The table gives the length of the periods, the average daily urinary nitrogen and acetone, the average amounts of foodstuffs estimated to have been metabolized daily, and the FA:G ratios. In order to determine the threshold of ketosis, the FA:G ratios are plotted against the average daily acetone excretion for each period (Chart III). From Chart III it appears that the threshold is reached when the FA:G ratio is approximately 1.5 for all the subjects except the obese man whose ratio is about 2.4. He never excreted more than 0.5 gm. of acetone bodies in 24 hours at any ratio observed.

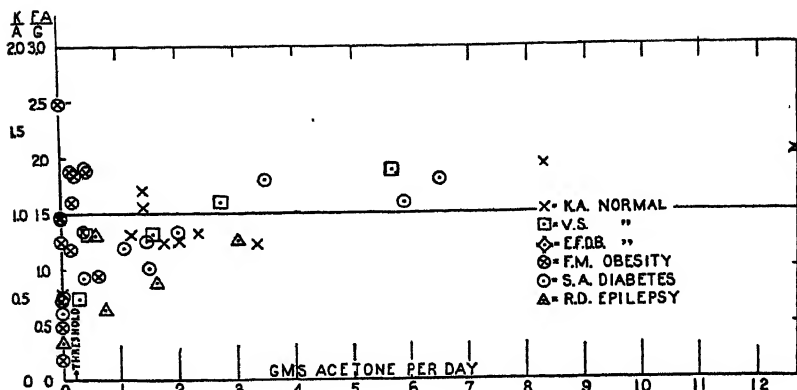


CHART IV. FA:G ratios of foodstuffs metabolized plotted against acetone excretion calculated from data obtained during calorimeter observations.

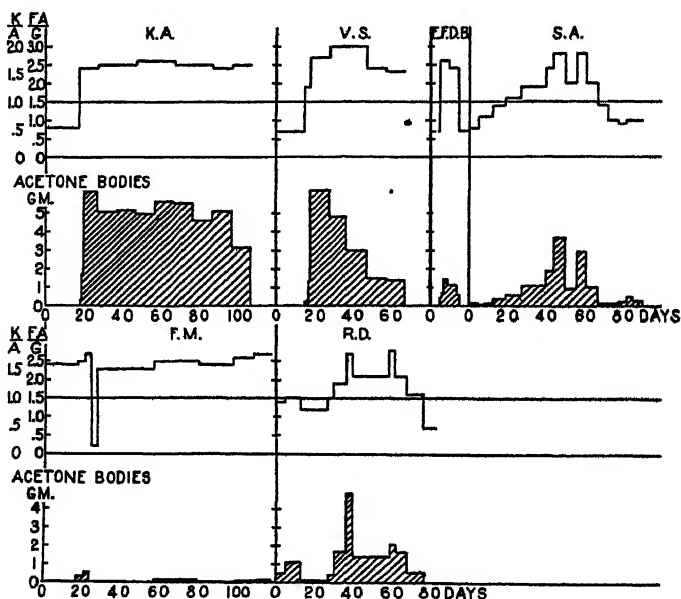


CHART V. Individual variations in the FA:G ratios of food metabolized and the average daily acetone excretion for the six subjects studied.



When the FA:G ratios are calculated from the calorimeter data and compared with the acetone bodies excreted during the same period (Chart IV), the threshold seems to be reached at a ratio of 1.0 except in the case of the obese man for whom no definite level was established. Acetone bodies excreted during the calorimeter periods were calculated as acetone per 24 hours.

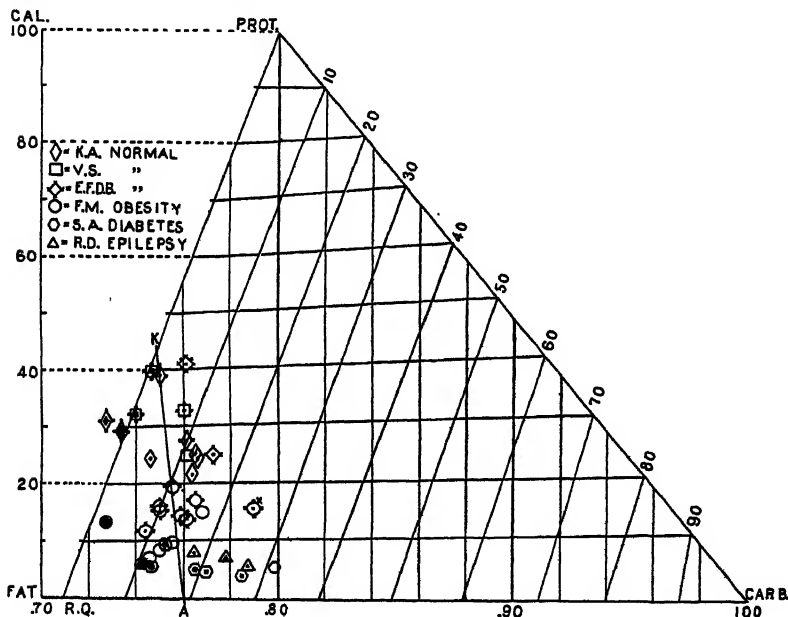


CHART VI. The triangle of Du Bois showing the zones of metabolism with individual calorimeter observations of 3 or 4 hours duration plotted. The plain symbols represent observations made under basal conditions and the tabbed symbols those made after meals. The size of the black center indicates the relative amount of acetone bodies excreted. In the observation marked X the subject derived 15 per cent of his calories from protein, 25 per cent from carbohydrate, and 60 per cent from fat.

The individual variations found in the different men studied are shown in Chart V. The subjects K. A., V. S., and E. F. D. B. received meat alone except for the first period. S. A. was a diabetic, F. M. an obese man, and R. D. an epileptic. A marked dissimilarity was noted in the acetone body production when they were

metabolizing foodstuffs with high FA:G ratios. When the men changed from a general diet to the meat diet it took 3 days before they were excreting relatively constant amounts of acetone bodies, a delay which was probably due to the use of reserve carbohydrate stored in the body. This reserve was practically exhausted by the end of the 3 day period. There was a diminishing ketosis in the subject V. S. The subject S. A. in Periods 8 and 10 metabolized foodstuffs with FA:G ratios of 2.8. His daily acetone excretion was 0.74 gm. less during Period 10. R. D. in Period 6 metabolized foodstuffs yielding an FA:G ratio of 2.7 and in Period 8 a ratio of 2.8, yet he excreted 2.87 gm. of acetone per day less during Period 8. These findings suggest that some adaptation to the use of fat took place in the diabetic and epileptic subjects.

Chart VI has a number of calorimeter observations plotted on the triangle devised by Du Bois (17) which shows the zones of metabolism. With it the nature of the foodstuffs metabolized can be determined from the urinary nitrogen and the calorimeter data. The observation, designated by X in Chart VI would indicate that the subject derived 15 per cent of the calories from protein, 25 per cent from carbohydrate, and the remaining 60 per cent from fat. The work of the past year has dealt mainly with the protein-fat portion of the triangle. The line KA indicates the location of the FA:G ratio of 1.5 and theoretically bounds the right side of that area of the triangle where we should expect ketosis to occur. Much work has been done below the 30 per cent protein line, but only with the men receiving purely meat diets were we able to explore the upper portion of this line. Individual observations of 3 or 4 hours have been plotted. The plain symbols represent basal observations and the tabbed symbols those made after food. The size of the black center indicates the relative amount of acetone bodies excreted during the calorimeter periods. The observations made on the obese man, even those lying to the left of the line KA, show little if any acetone, while observations made on the remaining subjects often show acetone, even those lying to the right of the line.

#### DISCUSSION.

When the carbohydrate reserve was exhausted, which required about 3 days in men who were taking diets very low in carbohy-

drate, we found the ratios very constant between the amounts of protein and fat metabolized even following meals when the total metabolism increased 10 to 20 per cent. The uniformity of the respiratory quotient was noted at different periods during the 24 hour cycle and although we did not obtain observations during the complete cycle we felt that no marked variation from this uniformity occurred. Since protein has a theoretical respiratory quotient of 0.801 and fat 0.707 we should expect that individuals subsisting mainly on these two foodstuffs would have quotients between these levels. Our findings verified this expectation and gave no evidence that either fat or carbohydrate was being formed in excess of the amount oxidized.

In regard to the variation in the threshold of ketosis reported by different investigators, it may be of value to consider what criteria have been used to determine the threshold. Wilder and Winter (8) speak of "clinically significant ketosis." They reported only three subjects who excreted less than 0.2 gm. of acetone per day on K:A ratios (Shaffer) above 1.0 (which corresponds to the FA:G ratio (Woodyatt) of 1.5). Ladd and Palmer (9), using a somewhat different formula  $F:G \left( \frac{\text{gm. fat}}{0.58 \text{ gm. protein} + \text{gm. carbohydrate}} \right)$

for calculating their ratios, reported that the threshold occurred when the ratio was about 4:1. In their data (Case XV) they found that the daily excretion of acetone bodies was between 0.1 and 0.4 gm. when the subject was on a diet of an F:G ratio of 1.95 which is comparable to an FA:G ratio of 1.63. It will be seen therefore that, when the data analyzed above are judged by the criteria previously mentioned for determining the threshold of ketosis, there is a general agreement among the observations of various workers regarding the FA:G ratio at which the threshold appears to lie. No effort is made here to show that the amount of acetone bodies excreted is related to the height of the FA:G ratio, yet this seems to hold during the early periods of each subject's observation.

We do not know why the threshold of ketosis for the calorimeter observations lies at FA:G ratios of approximately 1.0. Richardson and Ladd (7) (Table VI) found that when diabetic subjects were metabolizing foodstuffs with FA:G ratios between 0.8 and 1.2 they excreted 0.14 to 0.81 gm. of acetone, calculated from the

period of observation as acetone for 24 hours. Wilson, Levine, and Rivkin (10) located the threshold at a ratio of 1.0 for children on high fat diets. These observations and our own are the only ones of which we know where the foodstuffs metabolized have been calculated from 3 to 4 hour observations in metabolic chambers. It must be admitted that the acetone bodies excreted during a 3 or 4 hour period may not have been formed entirely from the food metabolized in that same period for there is possibly some lag in the excretion of acetone. However, during basal observations on our subjects, we noted that the lowest rate of excretion of acetone bodies for the day occurred 16 to 20 hours after food, so we feel that the acetone excreted may be compared fairly with the ratios of foodstuffs utilized for the same periods.

TABLE II.  
*Adaptation to Utilization of Fat in Case XV (Ladd and Palmer).*

Period No.	Date.	No. of days reported.	Diet received.			Ladd and Palmer ratio, F:G.	Wood-yatt ratio, FA:G	Average daily acetone in urine.
			Protein.	Fat.	Carbohydrate.			
	1923		gm.	gm.	gm.			gm.
4	Mar. 21-Apr. 7	13	50	247	53	3.00	2.30	1.59
5a	Apr. 13-27	11	50	264	17	5.74	3.60	2.80
5b	May 9-16	9	50	264	17	5.74	3.60	1.70
6	" 17-31	7	50	247	53	3.00	2.30	0.74

The amount of acetone excreted by the different men and by the same man at different periods varied. We have pointed out some of these variations in the preceding section which suggest that there is a diminishing ketosis in individuals who metabolize foodstuffs with high FA:G ratios for long periods. Ladd and Palmer from their data of Case XV made the same observation. We have summarized their data in Table II. Lennox and Cobb (18) in their monograph on epilepsy refer to "the adjustment to ketosis which the body is able to make." They say, "With a patient fasting or on a constant ketogenic diet, the plasma bicarbonate, which is at first decreased, gradually returns to normal, in spite of the presence of ketone bodies in both urine and plasma." Therefore the experimental findings give evidence that, in men taking

high fat diets for long periods, there is an adjustment or an adaptation on the part of the body so that it can burn fat more efficiently.

The obese man was able to utilize a high percentage of fat to complete his maintenance requirement without the formation of any significant amount of acetone bodies. His threshold was reached at FA:G ratios of about 2.4. Mason (14) in his studies of obesity found no acetonuria by the ferric chloride test. We have found in our work that this test is less sensitive than the sodium nitroprusside test. There is definite evidence that the obese man is strikingly different from all our other subjects. The explanation of this is not evident. He apparently uses ingested fat and body fat in the same way. A similar conclusion was reached by Hubbard (19) some years ago when he was studying ketogenic diets in normal men. Possibly the body of the obese individual has become adapted to the use of large amounts of fat over long periods of time so that ketosis is produced with difficulty. As the arctic explorers are to continue on the exclusive meat diet for about 6 months longer we shall be able to report if any further adaptation occurs.

#### SUMMARY AND CONCLUSIONS.

1. Observations on the threshold of ketosis, which is defined as the FA:G ratio (Woodyatt) of food metabolized at which an abnormal amount of acetone bodies first appears in the urine, have been made on six men. Three men received exclusive meat diets and the others very little carbohydrate so that all were taking relatively high ketogenic diets.

2. A marked uniformity in the level of the respiratory quotient was observed in men who received very low carbohydrate diets for long periods.

3. The threshold of ketosis when calculated for periods was found at the FA:G ratio of approximately 1.5 except for the obese man whose threshold was at about 2.4.

4. The threshold of ketosis during the calorimeter observations was reached with FA:G ratios of 1.0 for the general group. A higher but not definite level was noted for the obese man.

5. We have found no explanation for the difference in the thresholds of the obese man and the other individuals. We assume that

the obese man has developed a greater efficiency in the utilization of fat than the normal individual as evidenced by a minimal ketosis when he metabolized foodstuffs with high FA:G ratios.

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## CLINICAL CALORIMETRY.

### XLIV. CHANGES IN THE RATE OF EXCRETION OF ACETONE BODIES DURING THE TWENTY-FOUR HOURS.

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#### INTRODUCTION.

The importance of variations in the rate of excretion of acetone bodies, which rate may be taken as an index of the degree of ketosis in the body, has been emphasized by McQuarrie and Keith (1) in their work with epileptic children receiving high fat diets. They noted that the maximal rate of excretion occurred in the afternoon and the minimal during the morning hours when they received three equal meals at the usual meal hours. Forssner (2) found that men taking meat diets reached their maximal excretion during the night. Hubbard and Wright (3) found the rate highest in the afternoon.

Observations of the rate of excretion of acetone bodies were made at intervals during the course of detailed studies which were conducted in the metabolism ward of the Russell Sage Institute of Pathology upon two arctic explorers and one laboratory worker, while they were taking only meat in their diets.

#### *Methods.*

The total acetone bodies in each separate voiding of urine were determined for the 24 hour periods reported. The method of Van Slyke (4) was used and the amounts of acetone bodies calculated as acetone in gm. per hour. These men derived from 20 to 25 per cent of the food required for their maintenance from protein and the remainder from fat. They received their food in the usual three meals except as noted in the table. The acetone ex-

TABLE I.  
*Excretion Rate of Acetone Bodies in Urine during the 24 Hours.*

Date.	Time interval.	Urine volume.	Acetone bodies per interval.	Acetone bodies per hr.	N <sub>2</sub> in 24 hrs.	Foodstuffs metabolised per 24 hrs.			F.A. Grade.
						Protein.	Fat.	Carbohydrate.	
Subject K. A. (Maintenance requirement 2440 calories.)									
1928		cc.	gm.	gm.	gm.	gm.	gm.	gm.	
Jan. 31- Feb. 1*	7.00 -10.42 a.m.	175	0.78	0.21					
	10.42 a.m.- 2.54 p.m.	225	0.41	0.10	15.0	94	219	4	3.0
	2.54 p.m.- 7.00 a.m.	560	2.46	0.15					
Mar. 6-7	7.00 - 8.57 a.m.	150	0.16	0.08					
	8.57 a.m.- 1.10 p.m.	440	0.21	0.05	15.1	94	218	8	2.8
	1.10 p.m.- 7.00 a.m.	660	3.94	0.22					
Mar. 7-8	7.00 a.m.-12.22 p.m.	175	0.60	0.11					
	12.22 - 4.15 "	245	1.33	0.34	16.3	102	213	11	2.6
	4.15 p.m.- 7.00 a.m.	600	4.71	0.32					
Mar. 8-9*	7.00 -10.39 a.m.	190	0.47	0.13					
	10.39 a.m.- 2.41 p.m.	265	0.35	0.09	15.4	96	216	8	2.8
	2.41 p.m.- 7.00 a.m.	800	2.83	0.17					
Mar. 23-24†	7.00 -11.17 a.m.	185	0.23	0.05					
	11.17 a.m.- 2.11 p.m.	360	0.12	0.04					
	2.11 - 4.11 "	160	0.17	0.09	18.3	114	210	5	2.6
	4.11 p.m.- 7.00 a.m.	540	1.64	0.11					
Apr. 4-5	7.00 a.m.- 1.20 p.m.	250	2.00	0.32					
	1.20 - 6.10 "	250	0.68	0.14					
	6.10 -11.21 "	200	1.69	0.33	17.0	106	212	10	2.6
	11.21 p.m.- 4.35 a.m.	250	2.76	0.53					
	4.35 - 7.00 "	100	0.88	0.36					
Apr. 5-6	7.00 a.m.- 1.30 p.m.	220	0.46	0.07					
	1.30 - 5.50 "	210	0.51	0.12					
	5.50 -11.00 "	190	0.81	0.16	16.9	106	212	10	2.6
	11.00 p.m.- 7.00 a.m.	430	3.10	0.39					
Apr. 24-25	7.00 a.m.- 1.25 p.m.	220	0.20	0.03					
	1.25 -11.30 "	450	1.41	0.14	19.9	124	204	8	2.4
	11.30 p.m.- 7.00 a.m.	290	0.99	0.13					
Apr. 25-26	7.00 a.m.- 2.20 p.m.	250	0.18	0.03					
	2.20 -10.45 "	440	1.13	0.13	17.7	111	210	9	2.5
	10.45 p.m.- 7.00 a.m.	600	2.43	0.29					



TABLE I—*Concluded.*

Date.	Time interval.	Urine volume.	Acetone bodies per interval.	Acetone bodies per hr.	N <sub>2</sub> in 24 hrs.	Foodstuffs metabolised per 24 hrs.			FA: G ratio.
						Protein.	Fat.	Carbohydrate.	
1928									
Apr. 26-27	7.00 a.m.-12.45 p.m.	340	0.67	0.12	17.0	106	212	9	2.6
	12.45 - 4.50 "	440	1.64	0.40					
	4.50 p.m.-12.00 m.	420	1.15	0.16					
	12.00 m. - 7.00 a.m.	300	1.24	0.18					
May 27-28	7.00 a.m.- 3.30 p.m.	450	0.56	0.07	20.8	130	201	9†	2.3
	3.30 -11.10 "	650	1.15	0.15					
	11.10 p.m.- 7.00 a.m.	530	0.45	0.06					
May 28-29	7.00 a.m.- 3.00 p.m.	210	0.30	0.04	19.6	123	204	9†	2.4
	3.00 -11.30 "	380	0.63	0.07					
	11.30 p.m.- 7.00 a.m.	320	0.60	0.08					
Subject V. S. (Maintenance requirement 2680 calories.)									
Mar. 15-16	7.00 -11.46 a.m.	135	0.74	0.16	16.2	101	240	7	2.9
	11.46 a.m.- 3.42 p.m.	170	0.93	0.24					
	3.42 p.m.- 7.00 a.m.	640	1.77	0.12					
Apr. 12-13*	7.00 -10.15 a.m.	320	0.17	0.05	17.6	110	237	6	2.8
	10.15 a.m.- 2.22 p.m.	200	0.07	0.02					
	2.22 -11.40 "	420	0.52	0.06					
	11.40 p.m.- 7.00 a.m.	300	0.26	0.04					
Apr. 16-17	7.00 -10.55 a.m.	190	0.14	0.04	24.5	153	216	10	2.2
	10.55 a.m.- 6.35 p.m.	410	0.26	0.03					
	6.35 -11.27 "	240	0.07	0.01					
	11.27 p.m.- 4.30 a.m.	210	0.33	0.07					
Apr. 17-18	4.30 - 7.00 "	250	0.05	0.02	21.2	133	225	11	2.4
	7.00 a.m.- 3.00 p.m.	360	0.15	0.02					
	3.00 - 6.55 "	260	0.25	0.06					
	6.55 p.m.-12.00 m.	290	0.11	0.02					
	12.00 m. - 7.00 a.m.	300	0.85	0.12					
Subject E. F. D. B. (Maintenance requirement 2880 calories.)									
Apr. 18-19	7.15 -11.52 a.m.	280	0.10	0.02	22.1	138	244	10†	2.5
	11.52 a.m.- 1.41 p.m.	310	0.004	0.002					
	1.41 - 3.43 "	260	0.09	0.05					
	3.43 p.m.- 7.00 a.m.	750	0.27	0.02					

Regular meal hours 7.30 a.m., 12.30 p.m., and 6.00 p.m. The subjects received their meals at these hours except as noted here.

\* Two meals, at 8 and 7 p.m. † Two meals, at 10 a.m. and 6 p.m.

‡ Estimated on the basis of his average carbohydrate intake.

cretion through the lungs was not estimated. The methods used for estimating the daily requirement for maintenance and the kind and quantity of foodstuffs metabolized are described in the preceding article (5).

#### EXPERIMENTAL.

The data obtained are presented in Table I. The foodstuffs metabolized during the 24 hour periods and their FA:G ratios (Woodyatt) are included. Table II shows the quantities of acetone bodies excreted during calorimeter observations which were made from 16 to 20 hours after the latest ingestion of food.

TABLE II.  
*Excretion Rate of Acetone Bodies in Urine 16 to 20 Hours after Food.*

Subject.	Date.	Time interval.	Urine volume.	Acetone bodies per interval.	Acetone bodies per hr.
	1928		cc.	gm.	gm.
K. A.	Jan. 31	10.42 a.m.-2.54 p.m.	225	0.41	0.10
	Feb. 9	10.22 " -2.38 "	195	0.59	0.14
	" 24	10.10 " -2.23 "	260	0.25	0.06
	Mar. 8	10.39 " -2.41 "	265	0.35	0.09
	Apr. 13	10.04 " -2.05 "	210	0.29	0.07
V. S.	Apr. 12	10.15 a.m.-2.22 p.m.	200	0.07	0.02
E. F. D. B.	Apr. 19	9.59 a.m.-2.12 p.m.	180	0.13	0.03

There is considerable variation in the time when the maximal excretion of acetone bodies occurs, even in the same individual, but, when allowance is made for the variation in the time of meals, the findings are explained at least in part. The maximum hourly rate of excretion noted was 0.53 gm. per hour in the case of K.A. on April 4-5 between 11.30 p.m. and 4.30 a.m. The lowest rate for K.A. was 0.03 gm. per hour on April 25 between 7.00 a.m. and 2.20 p.m. The highest excretion usually was found in the late afternoon or during the night.

Marked differences in the quantities of acetone bodies excreted by our subjects occurred when they were metabolizing food mixtures of practically the same FA:G ratios. Full consideration of this point is presented in another article (5).

## DISCUSSION.

The rate of excretion of acetone bodies may depend first on the rate of metabolism during the day, second, on the nature of the foodstuffs utilized in the body, and third, on a possible lag in the elimination of the acetone bodies after they are formed.

Marked changes occur in the total metabolism during the day. The effects of the foodstuffs metabolized in the body and the physical activity of these men are the most important factors in this change. Our subjects exercised chiefly in the afternoon when they walked from 3 to 5 miles. The rest of the day they remained in the metabolism ward. The higher rates of acetone excretion followed a few hours after this period of exercise. The minimal excretion rates were reached during the calorimeter observations made from 16 to 20 hours after the latest ingestion of food, the time when the metabolism of these patients was basal.

As to the nature of the foodstuffs utilized by these men, we are able to exclude the carbohydrate factor almost entirely. All the observations were made 7 days or more after the ingestion of carbohydrate except such as may have been obtained from the glycogen of the meat consumed. Under these conditions the carbohydrate stores probably were well depleted. This idea is supported by the findings of repeated calorimeter observations where the subjects derived 5 per cent or less of their total calories from carbohydrate. In the absence of available carbohydrate, fat became the important foodstuff in maintaining the metabolism of the body. Fat was always available since it made up 75 to 80 per cent of their food intake in calories. No significant change was noted in the ratios between protein and fat utilized by the men in basal condition and after the ingestion of food (5). Krogh and Lindhard (6) and Rapport and Ralli (7) found no material change in the relative proportions of foodstuffs oxidized in the body during exercise when they were compared with those used by the subject in basal condition. Since no marked variation occurs in the ratio between protein and fat utilized by our subjects during the 24 hours whether after food or during exercise and since fat is the foodstuff available for oxidation, we should expect to find during those periods of day when extra calories were needed that the maximal amounts of acetone bodies would be formed.

There seems to be a lag in the excretion of acetone bodies because the maximal rates of excretion appeared a few hours after the periods of greatest physical activity. Also the minimal excretion rate continued through the forenoon although the men had taken the usual amount of fat as part of their morning meal. The lag may depend upon the rate at which the fat is absorbed from the intestinal tract as well as upon a retardation in the excretion of acetone bodies when formed.

#### SUMMARY AND CONCLUSIONS.

1. Observations on the rate of excretion of acetone bodies during the day were made on three men who were taking only meat as their diet.

2. The highest rate of excretion occurred during the late afternoon and night while the lowest rate was in the forenoon. These findings agree with those of other observers.

3. The explanation for this characteristic variation includes a consideration of the rate of metabolism during the day, the nature of foodstuffs utilized in the body, and the possible lag in the elimination of the acetone bodies after they are formed.

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## BILE SALT METABOLISM.

### I. CONTROL DIETS, METHODS, AND FASTING OUTPUT.

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These experiments are a logical continuation of the research programs of Foster and Hooper (2), Wisner (8), Smyth (5), and Whipple,—a comprehensive study of the physiology of bile salts as they are formed within the body, secreted, and reabsorbed. Since much of the older literature was discussed in these papers as well as in a general review (6) of this subject this need not detain us at this time.

These experiments also give needed information about the open bile fistula as compared with a certain type of closed bile fistula. The experiments noted above reported by Foster, Hooper, Wisner, Smyth, and Whipple were all done with the familiar open bile fistula. Such dogs have an obstructed common bile duct and an open fistula from the gallbladder through the right rectus and by proper technique bile can be quantitatively collected. On suitable diets such dogs may remain in normal health and activity for months or even years. Although the fistula is infected it does not cause impairment of health as long as the drainage is continuous and the diet carefully regulated. The presence of infection may cause interference with certain studies of bile pigments and is always an unpleasant factor, to be viewed with suspicion.

We decided therefore to use the closed sterile fistula method of Rous and McMaster (4) whereby the bile is kept sterile and collected continuously over 8, 12, or 24 hour periods. This method like all others in this difficult field is far from perfect and we note at times uncontrollable fluctuations which in many instances are to be explained by kinks where the cannula is tied into the bile duct, or by bits of mucus or precipitate forming a plug which may tempo-

rarily slow or obstruct the flow through the tubing. Frequent bile cultures were taken during all experiments and the tables show whether the bile was sterile or infected.<sup>1</sup>

Infection with common air-borne spore bearers (for example *Bacillus subtilis*) does not cause any clinical disturbance and our experiments show that the bile salt metabolism is unchanged by the presence of these non-pathogenic bacteria in the bile passages, even with the closed fistula.

The experiments tabulated below indicate clearly that there are endogenous and exogenous factors concerned in bile salt metabolism. The level of bile salt excretion is less in sugar feeding days than in simple fasting (2), indicating an important relationship between the body protein breakdown and the bile salt output. From every point of view the bile salts are evidently related to protein breakdown—*endogenous* in these fasting experiments and *exogenous* or related to food protein digestion, as noted in the meat products feeding experiments tabulated in Paper II.

This paper includes data (Table 1) to show the bile salt and bile volume output per 8 or 12 hour periods. These experiments show similar figures to those noted in the paper of Whipple and Wisner (8). The output figures during daytime, evening, or night are almost identical when one averages the individual fluctuations of dogs on special or mixed diets. That the bile salt output is so little influenced by the time of food intake is somewhat surprising. It becomes obvious from a study of many tables given in Paper II that there is a distinct lag in output when a dog is fed a diet most favorable for bile salt production. This lag may amount to 24 to 48 hours in the curve of production before the maximum bile salt output is recorded:

Furthermore, after this favorable diet period there may be a "carry over" into a sugar feeding or unfavorable diet period so that the low sugar level may not be attained within 3 or 4 days (Table 23, Paper II). This indicates that this bile salt metabolism must be somewhat complex—not a simple protein splitting but a re-grouping of amino acids with synthesis. All this takes time and may explain at least in part the fact that the hour of feeding does not modify the output of bile salt in the subsequent 8 hour

<sup>1</sup> We are indebted to Dr. J. A. Kennedy of the Department of Bacteriology for these cultures.

collection period. The question of storage of bile salt factors during favorable diet periods and subsequent depletion of such stores when need arises is discussed in Paper II and probably explains a part of the reaction.

### *Methods.*

The bile fistula operations were performed after the method of Rous and McMaster (4). The gallbladder is removed and a glass cannula is tied into the common bile duct. From it the bile is led through about 20 cm. of rubber tubing of 3 mm. inside diameter and 1.5 mm. wall, and is brought to the outside through a stab wound in the right flank. The tube is joined to one arm of a 3-way glass connection which connects to a rubber balloon of 350 cc. capacity and by the other arm to an outlet tube which terminates in a glass tip and can be flamed before the bile is removed from the bag. Between collections this tip is kept covered with a rubber cap made by tying a bit of glass rod into one end of a short piece of fine rubber tubing. A number of these rubber caps are made up in advance and are autoclaved separately in Petri dishes. After the cap is placed over the glass tip a part of the cap and the exposed portion of the glass tip are wrapped with iodoform gauze so that at the time of the next collection the cap may be withdrawn without danger of organisms being introduced into the lumen of the glass tip. Several sterile sponges are fastened about the rubber tubing where it emerges from the stab wound. A little vaseline applied to the lower of these helps keep the sponge in close apposition to the wound and helps to prevent access of organisms along the course of the tubing. The rubber bag and sponges are covered over and protected by a light oval helmet-like copper basin thickly padded about the edges to prevent irritation and to give sufficient depth to prevent any possibility that the rubber bag underneath might be compressed and the free flow of bile impeded. This copper basin is about 20 by 10 cm. in diameter and when the edges are built up with padding is about 8 cm. deep in the middle. We found this arrangement superior to the grass baskets used by Rous and McMaster for, in our experience, the baskets are easily destroyed and now and then one of the stems wears loose and punctures the rubber bag, causing contamination of the contents, and, secondarily, generalized infection of the bile passages in the liver.

The copper basin is held in place by a canvas sling, the two terminal tails of which pass through small metal rings soldered to the top of the basin. A canvas jacket is then fitted to the dog. The jacket is tied up the back and is held from slipping by being provided with armholes for the front legs. The jacket is made to bulge outward over the metal basin to fit very snugly about the edges of the latter. If the jacket is properly made it holds the basin securely in place and allows one to dispense with the objectionable sheet of adhesive tape passed over the back of the dog by Rous and McMaster to anchor the basket in place.

Female dogs were used in all experiments so as to minimize the soiling of the jacket by urine. The bile is collected once a day, or oftener if desired, and with proper precautions sterile bile can be obtained from the dog several months before accumulations of bile salts, cells, or mucus stop up the cannula in the common bile duct or before accidental contamination allows access of organisms to the bile passages.

The need of careful and constant supervision of these bile fistula dogs cannot be too much emphasized. This requires almost the entire time of a skilled technician who weighs and observes the clinical condition of the dog. He takes the greatest care with the sterile bile collections which must be done with painstaking aseptic technique. Administration of any materials by stomach tube calls for skill and attention to subsequent feeding to minimize the tendency to vomiting. The whole day's existence of the dog must be under his eye and the slightest departure from normal is at once brought to the attention of one of the writers.

The standard bread is crumbled into equal weights of water after which the canned salmon is well mixed with it into a sort of mash. This prevents the dog from picking out salmon meat alone. The figures in any table refer to the amounts of bread and salmon actually eaten. In all instances the mixtures contain 10 gm. of whole standard bread for each gm. of canned salmon.

The method of Foster and Hooper (1) for the analysis of bile salt in bile has been modified in several respects, though the general principles involved are the same. Larger quantities of material have been used, thus increasing greatly the precision. The modified technique is given in detail below. The bile salt is estimated from its content of amino nitrogen as determined by the method



of Van Slyke. As a preliminary step the taurine of bile salt must be freed from the cholic acid by hydrolysis, otherwise its nitrogen is not set free in the reaction chamber of the apparatus. Since the bile contains small amounts of certain substances having free amino groups it is necessary to determine these by a preliminary analysis carried out on unhydrolyzed bile. This value is deducted from the amount obtained after hydrolysis, the difference being the nitrogen derived from the taurine of the bile salt. The purpose of the preliminary treatment with hot alcohol is to remove the mucins and any other protein substances which may be present in the untreated bile.

As a preliminary step the bile is centrifuged to free it of any gross particles it may contain. 10 cc. of the bile are precipitated with 80 cc. of 95 per cent alcohol, heated on a steam bath to the boiling point, cooled, and made up to 100 cc. with alcohol. Filter. Two specimens of 40 cc. each are evaporated to dryness on the steam bath. One is washed out quantitatively with distilled water and is made up to 10 cc. with the latter. 3 cc. samples of this are used to determine the amount of amino nitrogen present before hydrolysis. The other specimen is washed out quantitatively with 6 cc. of 8 per cent sodium hydroxide into a 10 cc. volumetric flask. The flask is stoppered with a large piece of rubber tubing partially closed at the top with a tightly fitting piece of glass tubing drawn out to a tip so that evaporation will be minimized during the 6 hour period that the specimen is being hydrolyzed. The actual hydrolysis is carried out by allowing the bulb of the flask to hang down into the steam bath for this period. After the hydrolysis the contents of the flask are approximately neutralized by the addition of 0.5 cc. of 50 per cent acetic acid. The flask is then filled up to the 10 cc. mark with water. 3 cc. samples of this are used for the determination of the amino nitrogen in the Van Slyke micro apparatus. To obtain the mg. of bile acid nitrogen per cc. of bile the number of mg. of amino nitrogen in the 3 cc. sample of the hydrolyzed specimen minus the amount found to be present in the 3 cc. sample of the unhydrolyzed specimen is divided by the factor 1.20. To obtain total taurocholic acid the output of amino nitrogen is multiplied by the factor 36.7.

Interesting papers by Jenke, Enderlen, and Thannhauser (3) have just appeared. We have had no opportunity to use their

TABLE 1.  
*Salmon-Bread Diet, 8 Hour Collections.*

Dog 26-97.

Date.	Diet.	Hr. of collection.	Hrs. of secretion.	Vol. of bile.	Amino nitrogen.		Total tauro-cholic acid.
					In 1 cc. bile.	Total output.	
	<i>gm.</i>			<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1-21	Salmon-bread 440	8.35 a.m.	24	63.0	0.51	32	1180
	5.00-9.00 p.m.	4.30 p.m.	8	7.0	0.61	4.2	155
22	Refused food.	2.30 a.m.	10	22.5	0.61	13.2	505
		8.05 "	5.5	19.0	0.51	9.7	358
		4.10 p.m.	8	20.0	0.52	10.4	385
23	Salmon-bread 220	12.10 a.m.	8	9.0	0.69	6.2	230
	1.30-4.00 p.m.	8.00 "	8	0.0			
		4.00 p.m.	8	18.0	0.86	15.5	570
24	Salmon-bread 352	12.05 a.m.	8	14.5	0.54	7.8	288
	4.30 p.m.	8.00 "	8	9.5	0.62	5.9	217
		4.00 p.m.	8	0.0			
25	Salmon-bread 176	8.20 a.m.	16	33.0	0.71	24	890
	1.00-4.50 p.m.						
26	Salmon-bread 440	8.20 a.m.	24	37.0	0.28	10.4	385
	4.25-9.30 p.m.	4.10 p.m.	8	14.0	0.21	2.9	107
27	Salmon-bread 440	12.02 a.m.	8	25.0	0.40	10.0	370
	4.15-9.20 p.m.	8.00 "	8	21.0	0.49	10.3	380
		4.00 p.m.	8	20.0	0.57	11.4	422
		11.59 "	8	21.5	0.47	10.1	373
28	Refused food.	8.03 a.m.	8	20.0	0.68	13.6	500
29	Salmon-bread 303	8.04 "	24	25.0	0.56	14.0	515
	8.20-11.55 a.m.	4.00 p.m.	8	39.0	0.40	15.6	575
		11.58 "	8	38.0	0.34	13.0	478
30	Salmon-bread 440	8.04 a.m.	8	33.0	0.42	13.9	512
	8.30 a.m.-12.30 p.m.	4.00 p.m.	8	37.0	0.36	13.3	490
		11.57 "	8	36.0	0.37	13.3	490
31	Salmon-bread 83	8.05 a.m.	8	31.0	0.35	10.8	398
	9.30 a.m.-1.30 p.m.	4.00 p.m.	8	34.0	0.23	7.8	287
2-1	Salmon-bread 193	12.02 a.m.	8	24.0	0.21	5.0	184
	8.40 a.m.-12.40 p.m.	8.04 "	8	8.0	0.39	3.1	114
		4.00 p.m.	8	26.0	0.35	9.1	335
		11.59 "	8	13.5	0.45	6.1	225
Average values of 8 hr. periods.							
Day.....				21	0.46	9.0	332
Evening.....				23	0.45	9.5	350
Night.....				18	0.49	8.6	317

Bile fistula operation January 7. Bile sterile throughout. Weight during experimental period 11.5 to 11.2 kilos.

method but hope to report further on this point. Their tables do not include food intake, so that it is impossible to compare their figures with ours as the importance of food protein is not considered. Whenever given, the dog weight figures indicate a rapid loss of weight—for example, a loss from 20 to 14 kilos in 2 months. This means usually serious bile fistula infection or unsuitable food intake and introduces a very serious complication.

### *Experimental Observations.*

The bread used in these experiments (Table 1) is prepared in this laboratory for use with anemic dogs as described elsewhere (7). It consists of wheat flour, starch, bran, sugar, canned salmon, cod liver oil, canned tomatoes, yeast, and a salt mixture. It contains 1.16 gm. of nitrogen per 100 gm. This bread is mixed and baked by one of the technicians. It supplies a complete diet capable of maintaining an adult dog in health for long periods if not indefinitely. The bread is palatable and eaten readily but small amounts (10 per cent by weight) of canned salmon are added to the bread as an appetizer. The figures given in Table 1 show the amount of bread and salmon which was eaten daily.

Table 1 shows a continuous observation period of 11 days during which 8 hour collections were the rule. This dog did not eat as regularly as could be desired and there are 2 days on which it refused food. Such fasting days were followed by days of low output of bile and bile salts. However, the dog made up on diet intake on subsequent days and its weight showed only a decrease from 11.5 to 11.2 kilos. There are many days when the bile output is quite regular per 8 hour period and it may be noted that the average values show but little difference between the day, evening, and night collections. The night collections are very slightly below the day and evening periods. It is especially noteworthy that the output in the 8 hours following midnight is practically as great as at other times despite the absence of food and despite the fact that the dogs are at rest during this part of the day. The bile fistula was sterile during this entire period, yet on two occasions we note no collection during an 8 hour period. We must explain this by a transient obstruction due to a kink or to a small plug of mucus or sediment in the bile. Low diet intake is probably in part responsible for the low output of the last 2 days.

There are considerable fluctuations in bile concentration shown by the figures of amino nitrogen in 1 cc. of bile. Some factors which influence bile concentration will be discussed in the other papers of this series but we must admit that in general we cannot explain such variations as they occur from day to day.

Table 2 shows a continuation of observations on Dog 26-97 as in Table 1. The bile cultures remained sterile throughout. We observe the rapid fall in bile secretion and bile salt output when the

TABLE 2.  
*Fasting. Short Period Collections.*

Dog 26-97.

Date.	Wt.	Diet.	Hr. of collection.	Hrs. of secretion.	Vol. of bile.	Amino nitrogen.		Total taurocholic acid.
						In 1 cc. bile.	Total output.	
	<i>kg.</i>	<i>gm.</i>			<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
2-2	11.2	Salmon-bread 121	8.00 a.m.	24	44	0.43	19.0	700
3		" 314	8.05 "	24	62	0.38	23.5	865
4		Food withheld.	8.00 "	24	54	0.50	27.0	995
5		" "	8.00 "	24	31	0.45	14.0	515
5		" "	11.57 p.m.	16	13	0.66	8.6	317
6		" "	11.00 a.m.	12	4.9	0.74	3.6	132
7		" "	12.02 "	12	6.5	1.32	8.6	317
7		" "	8.50 "	8	3.0	1.10	3.3	122
7		" "	11.57 p.m.	15	2.6	1.50	3.9	144
8	10.0	" "	8.05 a.m.	8	1.4	2.10	2.9	107
Average values per hr. during fast.								
Day and evening.....						0.40	1.02	18.2
Night and morning.....						0.24	1.10	9.5

Bile fistula operation January 7. Bile sterile throughout.

dog is fasting, yet there is a considerable "carry over" from the bread period into the fasting period so that the fasting level is not reached until the 3rd or 4th day without food. We note a great concentration of bile salts in whole bile during fasting so that the fasting bile contains 3 or 4 times as much bile salt per 1 cc. of bile. It is clear that in this experiment the night output falls below the day output. We may note the following facts as bearing on this observation. There is no change in bile concentration but only in

bile salt output. We are dealing with very small volumes of concentrated bile and we may argue that bodily activity may favor the outflow of this syrupy bile sufficiently to explain these differences. We hesitate to believe that the fasting organism produces more bile salt during moderate activity than during rest but prefer to think of activity squeezing out more completely from the biliary tree this thick syrupy concentrated bile.

#### DISCUSSION.

We wish to compare these experimental periods (Tables 1 and 2) with those in subsequent papers—for example, Tables 21 and 27 (Paper II). These other diet periods with standard bread and salmon give a remarkably constant total bile salt output—about 100 mg. of bile salt per kilo of body weight per 24 hours. This gives us a fixed base-line and the evidence is strong that the production is pretty uniform throughout the whole day, regardless of the hours of feeding or sleep. If anything is to be said it is to the effect that the early morning hours may show a very small decrease as compared with the early evening bile salt output when digestion is at its height.

These observations are entirely in harmony with those of Wisner and Whipple (8) who made 24 hour collections subdivided into 6 hour intervals. We may compare our experiments on a diet of standard bread plus salmon with their experiments in which a diet of potato, rice, and milk was used. The output per kilo per 24 hours is very close to 100 mg. Wisner and Whipple (8) concluded that there was little if any difference between the various 6 hour periods and if anything could be said it was to the effect that the night bile output might be slightly less than the normal day output. This shows absolute parallelism between the closed sterile fistula and the open infected fistula in this type of experiment. In comparing the fasting and sugar feeding experiments in this and subsequent papers we may refer to the older experiments of Foster, Hooper, and Whipple (2). During fasting or sugar feeding (Table 2, and Tables 23 (Paper II) and 32 (Paper III)) we note very low values for bile salts—about 300 to 400 mg. of bile salt per 24 hours or 30 to 40 mg. per kilo of body weight per 24 hours. Foster, Hooper, and Whipple (2) record higher values or about 100 mg. of bile salt per kilo of body weight per 24 hours, for fasting dogs and

about 80 mg. of bile salt per kilo of body weight per 24 hours for sugar-fed dogs.

In correlation of these results we may submit the following argument. The older experiments of Foster, Hooper, and Whipple (2) were done with an open fistula which we believe introduces no change in bile salt production, but their experiments dealt with 6 hour collections and the calculation for 24 hours is given on this basis. We know that the fasting bile is concentrated and syrupy and some bile could be licked from the open fistula during night periods of non-collection. Also these open fistula dogs were set up each morning to drain for 30 minutes to permit any excess of accumulated night bile to escape before the 6 hour collection was begun. It is possible, if not probable, in view of the data here given (Table 2) that the syrupy night bile did not completely drain during this 30 minute preliminary drainage and therefore increased the 6 hour output.

It is obvious that fasting will reduce the output of bile and bile salt to low levels. The output of bile salt in our experiments cannot be reduced below 30 to 40 mg. of bile salt per kilo of body weight per 24 hours. This we may term the base-line of *endogenous* bile salt production and as it continues over long periods of time we must assume that this bile salt must come from body tissue breakdown. The fact observed by Foster, Hooper, and Whipple, that the output of bile salt decreases with sugar feeding as compared with fasting periods, is further evidence that body protein breakdown is the source of some fraction which is built into the finished bile salt within the liver.

We note also in sugar feeding (Table 23, Paper II) or fasting that the bile salt output falls slowly over a period of 2 to 3 days when the sugar diet or fasting begins. Again when the dog is given a favorable diet following a period of sugar feeding or fasting there is a slow rise during 2 to 3 days to the expected favorable diet level. This may indicate a certain storage of materials suitable for bile salt production to be gradually depleted as fasting continues. When a favorable diet follows fasting we may postulate a storage of a part of this material which will not appear as bile salt until subsequent emergency needs arise.

## SUMMARY.

Bile salt secretion in dogs is remarkably uniform during the 24 hours in spite of a single daily feeding.

Daytime (8.00 a.m. to 4.00 p.m.) or evening (4.00 p.m. to 12.00 m.) or night-time (12.00 m. to 8.00 a.m.) all show practically equivalent bile salt output. There may be a very slight difference—lower at night-time during the period of bodily quiet, when compared with the evening collection which includes maximal digestive activity.

The base-line output of bile salt on a diet of standard salmon bread is very close to 100 mg. per kilo of body weight per 24 hours. Individual dogs may show values as low as 80 or as high as 130 mg., but this level is fairly constant for any given dog.

Fasting reduces the bile salt output to very low levels. Sugar feeding likewise shows very low levels, even lower than fasting.

Bile salt output continues even during long fasting periods. This fasting base-line is about 30 to 40 mg. of bile salt per kilo of body weight per 24 hours. This represents the *endogenous* portion of the bile salt formation. There is good reason to believe that the breakdown of body protein contributes an essential component for such bile acid synthesis.

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## BILE SALT METABOLISM.

### II. INFLUENCE OF MEAT AND MEAT EXTRACTIVES, LIVER AND KIDNEY, EGG YOLK AND YEAST IN THE DIET.

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It is clearly apparent from a study of the tabulated experiments given below that meat products increase notably the output of bile salts and whole bile. These experiments confirm and extend those of Foster, Hooper, and Whipple (2). There are no differences to be observed between the open and closed bile fistula in these varied experiments.

It becomes more certain that food *proteins* are concerned with this large increase in bile acid output—this being an *exogenous* factor in bile salt metabolism. We note that beef skeletal muscle and liver feeding give the highest outputs of bile salt, while kidney and beef heart are a little less potent. It has been observed (2) that there is an interesting parallelism between the urinary nitrogen and bile salt output. For example the bile salt output with fasting is very low but it may be further reduced by sugar feeding which necessarily decreases the urinary nitrogen (protein-sparing action). Likewise with increasingly heavy beef feeding (2) the bile salt output will increase proportionally to the diet intake and related urinary nitrogen. All this evidence from all sides points to *protein breakdown* (body protein or food protein) as the source of an essential radicle for the synthesis of bile acids within the liver.

*Cholesterol* and possibly other related lipids have long been suspected of being the source of bile acids. This is largely due to similarities of chemical structure which are always intriguing. This illustrates again the well recognized fact that from the chemical formula no accurate specifications can be written about any chemical substance as to its reaction or fate in the living body. The

literature on this subject has been reviewed recently (3), and experiments reported dealing with cholesterol feeding.

It seemed wise in our experiments tabulated below to use egg yolk rather than pure cholesterol to insure complete absorption. Bile salts were added to secure more complete and normal absorption (Table 27). These experiments furnish strong evidence that, at least in the dog, cholesterol is not concerned in any way with bile salt output.

Furthermore the lipids extracted from meat by hot alcohol were shown to be inert but the lipid-free meat protein was as potent as usual when tested in the bile fistula dog to increase bile salt output (Table 24). Laked red cells and stroma have also been given intravenously (3) and by mouth with no effect. It has been repeatedly suggested and claimed that the red cell stroma rich in cholesterol was the main source of bile salt production in the body. So it would seem that in spite of the very conspicuous structural similarities, bile acids and cholesterol (dogs) are innocent of any metabolic relationship.

#### *Experimental Observations.*

The methods have been described in Paper I of this series. The meat products were all prepared in the same manner for the daily feedings. The meat or organ tissues were cut up and boiled in water. The water in which the boiling was done was saved and evaporated to a thick broth which was added to the meat or organ tissue before feeding, thus insuring against loss of potent water-soluble extractives. The amounts of food given in the tables represent the total consumption and indicate about the maximum which the dog will eat. In the case of meat diets, the amount eaten as indicated in the tables is the weight of the fresh meat products before cooking. Such fresh meat contains approximately 20 per cent protein. The body weights remain fairly constant throughout. It is well recognized that dogs with all bile diverted from the intestine cannot digest as completely various food materials and demand a higher food intake.

With an occasional exception all these tables present analyses of material from sterile bile tracts. The infected bile tracts (Tables 21 and 27) show no evidence of change in bile salt output. The dogs show no clinical disturbance in the usual case where air-borne

spore bearers (for example *Bacillus subtilis*) are found in the bile and we feel it safe to assume that this type of infection introduces no significant complicating factor. If the dog becomes infected

TABLE 21.

*Beefsteak, Beef Heart, Kidney, Liver, and Salmon-Bread Diets.*

Dog 26-162.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
				In 1 cc. bile.	Total output.	
	<i>kg.</i>	<i>gm.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
11-12	15.8	Salmon-bread 253	110	0.30	33	1220
13	15.6	" 292	104	0.39	41	1510
14	15.4	" 330	103	0.47	48	1770
15	15.7	Beefsteak 670	119	0.37	44	1630
16	15.8	" 670	180	0.39	70	2580
17	15.6	" 570	192	0.49	94	3470
18	15.8	" 570	172	0.52	89	3280
19	15.6		168	0.50	84	3100
12-22	14.8	Veal kidney 800				
23	14.5	" " 800	134			
24	14.7	" " 800	142	0.45	64	2370
25	14.6	" " 800	171*	0.45	77	2850
26	14.8	" " 800	164	0.45	74	2730
27	15.6		156	0.44	69	2560
1-3	15.6	Beef heart 800	97			
4	15.3	" " 800	144	0.41	59	2180
5	15.2	" " 800	156	0.47	73	2700
6	15.2	" " 800	110	0.64	70	2580
7	15.0		135	0.54	73	2700
10	15.3	Pork liver 800	168	0.42	70	2580
11	15.3	" " 800	166	0.56	93	3440
12	15.3	" " 800	147	0.57	84	3110
13	15.2		142	0.58	82	3030
22	15.3	Salmon-bread 440	76	0.48	37	1370
23	15.5	" 440	124	0.41	51	1890
24	15.5	" 440	134	0.32	43	1590
25	15.6	" 440	146	0.30	44	1620
26	15.9	" 440	156	0.29	45	1660
27	16.1		158	0.29	46	1700

24 hour bile collection made at 8.00 a.m.

Bile fistula operation November 2.

Bile culture first positive January 13.

with a pathogenic microorganism we note at once a serious clinical disturbance with loss of appetite and weight.

Table 21 gives a typical illustration of meat products feeding in a standard closed bile fistula which was kept sterile during 2½ months.

TABLE 22.  
*Pork Liver, Beefsteak, and Salmon-Bread Diets.*

Dog 26-97.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
			In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	mg.
2-12	Pork liver 700	74	0.33	24	885
13	" " 700	98	0.57	56	2070
14	" " 700	126	0.69	87	3200
15	" " 700	116	0.76	88	3240
16	" " 700	98	0.85	83	3050
17	Salmon-bread 275	59	1.03	61	2250
18	" 440	29	1.29	37	1360
19	" 275	31	1.37	42	1550
20	" 352	32	1.08	35	1290
21	" 385	54	0.72	39	1440
22	" 314	61	0.59	36	1330
3-4	Beefsteak 550				
5	" 550	93	0.69	64	2360
6	" 550	126	0.65	82	3030
7	" 550	118	0.72	85	3140
8	" 220	114	0.74	84	3100
9	" 550	113	0.74	84	3100
10	Salmon-bread 319	101	0.78	79	2920
11	" 143	57	0.92	52	1920
12	" 153	40	1.07	43	1590
13	" 198	46	0.86	40	1470
14	" 396	68	0.43	29	1070
15		58	0.61	35	1290

24 hour bile collection made at 8.00 a.m.

Bile fistula operation January 7. Bile sterile throughout.

Weight during experimental period 10.8 to 10.1 kilos.

The bile infection noted in the last bread period causes no clinical disturbance, no loss of weight, and no change in bile salt output.

We observe that the bile salt output is almost exactly 100 mg. per kilo of body weight per 24 hours in both the fore and after sal-

mon-bread periods. A diet change to beef feeding causes a sharp rise within 48 hours to the beef feeding level of bile salt secretion—about 200 mg. per kilo per 24 hours. When the dog is given liberal diets of veal kidney or beef heart we note a slightly lower level or about 180 mg. of bile salt per kilo per 24 hours. Compare with kidney feeding figures in Table 28 where the output is somewhat higher—about 230 mg. of bile salt per kilo of body weight per 24 hours. Heavy liver feeding is of special interest and gives a bile salt output of about 210 mg. per kilo per 24 hours.

The bile volume is high in all experiments and the volume rises with meat products feeding but not enough to explain the bile salt increase. We note therefore increased concentration of bile salt per 1 cc. on meat, kidney, heart, or liver feeding as compared with the control salmon-bread diets.

It is surprising that the bile salt output is practically as high on beef muscle feeding as on pig liver feeding. One might suspect that the liver cells would contain some stored bile salts or their precursors and therefore give very high values when fed.

Table 22 contains further experimental data on meat and liver. The bile salt output in this smaller dog runs even higher than in Table 21. The beef muscle and pig liver diets give the high output levels of about 300 mg. per kilo of body weight per 24 hours. A change from the bread to meat or *vice versa* shows the corresponding bile salt output level attained within 48 hours, there being at least 24 hours lag after the change in diet. The salmon-bread output level is about 120 mg. per kilo per 24 hours, which is a bit above the average of 100 mg. This dog shows rather low volume figures on bread feeding, the bile salt concentration running even higher on the bread than on the meat feeding.

We may recall the interesting diet experiments of Foster, Hooper, and Whipple (2) who found in open bile fistulas on heavy beef heart feeding that the bile salt output ranged from 200 to 300 mg. per kilo of body weight per 24 hours.

Table 23 shows the results of feeding large amounts of a commercial beef extract which exerts very little influence upon the bile salt output. The bile salt output on standard salmon-bread runs above the average and is equivalent to 120 to 135 mg. per kilo of body weight per 24 hours. We observe very low levels on sugar feeding alone—25 mg. of bile salt per kilo of body weight per 24 hours.

Beef extract was given in large doses with sugar and water by stomach tube twice daily. We note a gradual fall from the salmon-bread level of bile salt output day by day to less than half—from a total bile salt output of 1800 mg. per 24 hours to 625 mg. per 24

TABLE 23.

*Liebig's Beef Extract, Cane Sugar, and Salmon-Bread Feeding.*

Dog 26-120.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
5-20	12.7	Salmon-bread 440	126	0.39	49	1810
21	12.7	" 440	130	0.39	51	1890
22	12.7	" 440	124	0.37	46	1700
23	12.6	Beef ext. 100, sugar 100	126	0.32	40	1470
24	12.6	" " 100, " 100	92	0.40	37	1370
25	12.5	" " 100, " 100	50	0.49	25	925
26	12.5	" " 100, " 100	42	0.57	24	885
27	12.4	" " 100, " 100	44	0.45	20	740
28		" " 100, " 100	30	0.56	17	627
29	12.5	Sugar 150	37	0.47	17	627
30		" 200	10	0.87	8.7	320
31	11.7	" 200	6	1.29	7.7	283
6-1	11.5	" 200	6.5	1.28	8.3	305
2	11.4	Salmon-bread 275	7	1.13	7.9	291
3	11.6	" 440	68	0.38	26	960
4	11.9	" 440	84	0.31	26	960
5	11.9	" 440	102	0.29	30	1105
6	12.4		112	0.34	38	1400

24 hour bile collections made at 8.00 a.m.

Bile fistula operation May 16. Bile sterile throughout.

Sugar solutions given 200 cc. each day in two doses, one at midday and the other in late afternoon.

100 gm. of beef extract contain approximately 10 gm. of nitrogen (Kjeldahl determination).

hours. The final level for beef extract feeding is about 50 mg. of bile salt per kilo of body weight per 24 hours.

These figures would indicate that in large amounts of commercial beef extract there are small amounts of material which the body can manufacture into bile salts. We may emphasize the fact that

the high bile salt output on meat diet is not related simply to the total intake of nitrogen in the food, for the meat extract contains approximately one-half as much nitrogen as does the whole meat

TABLE 24.

*Beefsteak, Fat-Free Fractions, and Fat Extracts of Beefsteak and Sugar Feeding.*

Dog 25-25.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
				In 1 cc. bile.	Total out- put.	
	kg.	gm.	cc.	mg.	mg.	mg.
4-15	8.3	Salmon-bread 110	18	0.91	16.5	605
16	8.3	" 220	24	0.76	18	660
17	8.5	" 242	21	0.88	19	700
18	8.6	Beefsteak 625	20	0.85	17	625
19	8.8	" 625	39	0.94	37	1360
20	8.7	" 625	60	0.96	58	2140
21	8.8	" 625	44	1.13	50	1850
22	8.8	Fat-free beefsteak 130	50	1.13	57	2100
23	8.7	" " 130	36	1.09	39	1440
24	8.5	" " 130	49	1.31	64	2360
25	8.4	" " 130	58	1.00	58	2140
26	8.4	" " 130	51	0.93	47	1740
27	8.2	Sugar 200	60	0.89	53	1960
28	8.0	" 200	24	1.25	30	1110
29	7.8	" 200	20	1.42	28	1030
30	7.7	" 200	17	1.42	24	885
5-1	7.7	" 200, fat ext. of steak 200	13.7	1.61	22	810
2	8.1	" 200, " " " 200	10.0	1.99	20	740
3	7.8	" 200, " " " 200	14.5	1.14	17	625

24 hour bile collection made at 8.00 a.m.

Bile fistula operation April 4. Bile sterile throughout.

Sugar solutions in 550 cc. of water given by stomach tube.

130 gm. of fat-free beefsteak contained 17 gm. of nitrogen (Kjeldahl determination).

diet, yet the bile salt output is only about one-fifth as great. In fact the output on meat extract is distinctly less than the output on salmon-bread which contains about the same amount of nitrogen. The experiments of Smyth and Whipple (4) with the open fistula

are in harmony with these observations. They used smaller doses of Liebig's extract (15 gm.) and observed no increase in bile salt output—also negative results with watery and alcoholic laboratory extracts of meat and liver.

The bile volume (Table 23) also shows interesting fluctuations. Sugar feeding alone always causes great shrinkage in volume and increase in salt concentration per cc. Large doses of beef extract exert a mild cholagogue action to offset the sugar concentration. The volume output on salmon-bread is a little larger than usual. These figures should be compared with Table 24.

Table 24 shows a striking contrast between the beef muscle freed from fat and lipids by alcohol extraction and the beef muscle fat extract. All the combined fats and lipids extracted from large amounts of chopped beef are quite inert when added to the standard sugar diet periods. The extracted meat residues freed of fats and lipids are quite as potent as whole meat to promote bile salt output in bile fistula dogs. The total amount of food substance in the fat-free diet is no more than in the fresh meat diet—both represent between 17 and 20 gm. of nitrogen daily intake.

This dog (Table 24) is rather smaller than the average and its salmon-bread diet output below the average—or about 80 mg. per kilo of body weight per 24 hours. The output on beef feeding is about 225 mg. per kilo of body weight per 24 hours and the output level on lipid-free beef residue is a little higher, or 260 mg. per kilo of body weight per 24 hours. We may choose to explain this increase as due to the increased digestibility of the alcohol-extracted meat, as it is well known that fat makes protein digestion somewhat more difficult in bile fistula dogs.

We note an unusual "carry over" into the sugar diet period. The level of bile salt output does not fall to the expected sugar feeding level within 4 days as is usually observed. In fact the level is not back even to the control salmon-bread level. It is difficult to explain this except by a "carry over" of unusual duration due to the preceding prolonged diet periods of heavy meat feeding. When the lipid extractives from the meat are added we note the same progressive fall as was observed in the preceding sugar feeding periods. A total of 625 gm. of steak was fed daily but this represents the weight of the uncooked meat. During cooking much water is lost and the weight of the ground boiled meat is only about 300 gm.



daily. From April 22 the dog received an exclusive diet of finely ground beefsteak which had been thoroughly extracted with hot alcohol. The 130 gm. represent dry weight which was obtained from about 625 gm. of fresh steak. The details regarding the extraction of fat from the meat are given by Sperry (5). In brief, 10 pounds of lean steak are trimmed free of fascia and excess of fat, after which the meat is ground in a meat grinder and mixed with about 2 volumes of hot alcohol and is brought to boiling tempera-

TABLE 25.  
*Egg Yolk and Salmon-Bread Feeding.*

Dog 27-70.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
			In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	mg.
1-8	Salmon-bread 286	122			
9	" 308	138	0.36	50	1840
10	" 309	140	0.36	51	1880
11	" 418	140	0.36	49	1810
12	" 418	122	0.36	44	1620
13-15	" diet.				
16	Egg yolk 90, salmon-bread 275				
17	" " 90, " 176	99	0.38	38	1400
18	" " 90, " 187	95	0.40	38	1400
19	" " 90, " 280	81	0.44	36	1330
20		73	0.47	36	1330

24 hour bile collection made at 8.00 a.m.

Bile fistula operation January 5. Bile culture first positive January 20.

Weight during experimental period 12.8 to 13.8 kilos.

ture. The alcohol is strained off and the ground meat is extracted for 5 hours with hot alcohol vapors by a special reflux condenser. According to Bloor (1) this procedure removes practically all of the lipid substances. The fatty materials extracted in this manner from steak were also fed to the dog but they were totally inert as regards bile salt production (May 1 to 3). The fat in rather large amounts (from 1100 gm. of steak) was fed to the dog daily together with large amounts of cane sugar.

Table 25 illustrates very well the fact that cholesterol feeding

does not influence the output of bile salts in bile fistula dogs. This dog shows a rather high bile salt output on the standard salmon-bread diet—about 130 mg. of bile salt per kilo of body weight per 24 hours. A change to heavy egg yolk feeding shows no increase in bile salt output but rather a distinct decrease to the level of about 100 mg. of bile salt per kilo of body weight per 24 hours. We note also a decrease in bile volume and loss of weight during the egg yolk feeding period. These changes are not noted in the two subsequent experiments.

TABLE 26.  
*Egg Yolk and Salmon-Bread Feeding.*

Dog 27-79.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
			In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	mg.
2-5	Salmon-bread 154	90			
6	" 187	87	0.34	30	1105
7	" 275	77	0.36	28	1030
8	" 220	80	0.31	25	920
9	" 275	75	0.36	27	995
10	Egg yolk 90, salmon-bread 170	67	0.39	26	960
11	" " 90, " 170	84	0.38	32	1180
12	" " 90, " 165	84	0.34	29	1070
13	" " 90, " 154	94	0.39	37	1360
14		81	0.34	28	1035

24 hour bile collection made at 8.00 a.m.

Bile fistula operation January 30. Bile sterile throughout.

Weight during experimental period 9.7 to 9.4 kilos.

Table 26 also gives more evidence that large amounts of cholesterol (egg yolk) do not modify the output of bile salts under the conditions of these experiments. The 90 gm. of egg yolk represent the cooked egg yolk of five eggs. The standard salmon-bread output level is 100 mg. of bile salt per kilo of body weight per 24 hours. There are no significant changes in body weight, bile volume, or bile salt concentration.

Table 27 is much like Table 26 in all respects. We note the control salmon-bread level as 100 mg. of bile salt per kilo of body weight per 24 hours. We added 3 days of taurocholic acid feeding

plus the egg yolk feeding to give even more assurance of effective cholesterol absorption. It is readily seen that the bile salt output is not modified by the bile salt feeding except for the expected addition of this ingested bile salt to the base-line bile salt output. The bile salt presumably favors the absorption of egg yolk cholesterol but these experiments give no evidence that the cholesterol is related in any way to the bile salt output.

TABLE 27.

*Salmon-Bread Feeding Supplemented by Egg Yolk and by Egg Yolk and Bile Salt.*

Dog 26-162.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
			In 1 cc. bile.	Total output.	
	<i>gm.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1-22	Salmon-bread 440	76	0.48	37	1370
23	" 440	124	0.41	51	1880
24	" 440	134	0.32	43	1580
25	" 440	146	0.30	44	1620
26	" 440	156	0.29	45	1660
27	Egg yolk 90, salmon-bread 340	158	0.29	46	1700
28	" " 90, " 340	154	0.35	54	1990
29	" " 90, " 340	120	0.42	50	1850
30	" " 90, " 340	142	0.34	48	1770
31	" " 90, " 340	150	0.30	45	1660
2-1	" 80, taurocholate 0.4, salm.-br. 340	155	0.31	48	1770
2	" 80, " 0.4, " 340	181	0.33	60	2210
3	" 80, " 0.4, " 340	186	0.32	59	2170

24 hour bile collection made at 8.00 a.m.

Bile fistula operation November 2. Bile culture positive since December 13.

Weight during experimental period 15.3 to 16.2 kilos.

This experiment has an added interest because of the presence of infection of the bile passages. There was no clinical disturbance and there was a regular normal bile flow. The dog's weight on January 22 was 15.3 kilos and on February 4 was 15.8 kilos with minimal fluctuations. This emphasizes again the negligible factor of infection of the bile tract by non-pathogenic organisms, even with a closed fistula.

Table 28 gives data on yeast feeding. It was of interest to exclude yeast nucleic acids as related to bile salt metabolism. To the standard salmon-bread diet were added 50 gm. of pressed yeast (Fleischmann's) but we observe no change in the bile salt output. There are no significant changes in the bile volume or bile salt concentration. The body weight is constant and the bile passages sterile. The kidney diet output level is a little higher than that observed in Table 21.

TABLE 28.  
*Yeast, Kidney, and Salmon-Bread Feeding.*

Dog 27-44.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
			In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	mg.
12-14	Yeast 50, salmon-bread 358	126			
15	" 50, " 440	102	0.51	52	1920
16	" 50, " 440	113	0.41	46	1700
17	" 50, " 440	94	0.43	40	1470
18	" 50, " 440	87	0.45	39	1440
19	Salmon-bread 440	90	0.53	48	1770
20	" 440	87	0.53	46	1700
21	" 440	100	0.45	45	1660
22	Kidney 800	85	0.61	52	1920
23	" 800	126			
24	" 800	151	0.42	63	2320
25	" 800	145	0.51	74	2730
26	" 800	196	0.43	84	3100
27	" 800	142	0.54	77	2840

24 hour bile collection made at 8.00 a.m.

Bile fistula operation October 20. Bile sterile throughout.

Dog had been on bread diet since December 10.

Weight during experimental period 12.7 to 13.5 kilos.

#### DISCUSSION.

One reads constantly in the literature of the "well known" or "firmly established" relationship between the body *cholesterol* and bile salt production. This is an attractive hypothesis based largely on the careful and laborious chemical studies of Windaus who has shown clearly that there is a striking similarity of structure of

cholesterol and cholic acid. Physiological support for this hypothesis is conspicuous by its absence and we feel that the evidence is now conclusive that cholesterol and alcohol extractives have nothing to do with the varied bile salt fluctuations in the dog. The physiological evidence moreover is becoming more convincing that *proteins* are essentially related to these bile salt fluctuations. Both body protein and food protein seem to be concerned—endogenous and exogenous factors. There seems to be no direct relationship between bile salt output and the caloric value of the different diets. The salmon-bread is rather rich in fats with the result that the total intake per day is usually between 1000 and 1500 calories. The caloric value of the meat diet usually approximates 700 to 800 calories, and yet the bile salt output is vastly greater on meat diets. On Liebig's meat extract and sugar the caloric intake is also about 700 and on this diet the intake is even less than on salmon-bread.

These experiments show beautifully the lag which follows any change of diet whether from favorable (meat) to unfavorable (bread or sugar) or *vice versa*. We have discussed some of the possibilities which may be concerned: a storage of these bile salt factors during favorable diet intake and release during unfavorable diet periods; a slow metabolic turnover because of complex resynthesis of bile salts from amino acid groups.

#### SUMMARY.

Meat and meat products, including beef heart, kidney, and liver, when fed to bile fistula dogs cause a notable rise in bile salt output. This increase is usually a 2- or 3-fold increase above the standard salmon-bread bile salt output.

Cholesterol (egg yolk) feeding with or without bile salt causes no increase in bile salt output.

Alcoholic extractives of meat cause no increase in bile salt output but the alcohol-extracted meat residue has lost none of its potency when fed to a bile fistula dog.

Commercial beef extracts may contain small amounts of materials favorable for bile salt output.

Yeast nucleic acid is not related to bile salt metabolism as its feeding gives no change in the bile salt output.

Lipids and cholesterol should now be put aside, even if with

regret, as being innocent of any physiological relationship to the bile salt metabolism.

Food protein is of primary importance and determines the level of the exogenous bile salt output.

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## BILE SALT METABOLISM.

### III. TRYPTOPHANE, TYROSINE, AND RELATED SUBSTANCES AS INFLUENCING BILE SALT OUTPUT.

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(Received for publication, October 1, 1928.)

The experiments tabulated below give strong evidence that tryptophane<sup>1</sup> is an important determining factor in the output of bile salts under the conditions of these experiments. This fits satisfactorily with the observations on meat proteins given in Paper II. It is also in harmony with the observations of Foster, Hooper, and Whipple (1, 2).

*Gelatin* feeding gave significant figures for bile salt output in dogs as observed by Smyth and Whipple (3). When a diet of potato, rice, and milk was followed by gelatin feeding they observed no increase in bile salt output. This was quite different from experiments in which the potato, rice, and milk diet was followed by various meat proteins which always gave a marked increase in bile salt output. Smyth and Whipple realized the significance of this observation but were unable to continue the bile fistula work at that time.

We are able to confirm their observations that gelatin is notably inefficient for bile salt production—in fact its level is considerably below the standard salmon-bread level of bile salt output as established in many experiments given in Paper II. This suggests at once that tyrosine or tryptophane might be responsible for the low bile salt output on a gelatin diet. Supplementing gelatin with tyrosine gives negative results but supplementing gelatin with *tryptophane* at once raises the bile salt output close to the meat product output

<sup>1</sup> We are greatly indebted to Dr. Hans Clarke of the Eastman Kodak Company Research Laboratories, who prepared liberal amounts of pure tryptophane for these experiments.

level. Other chemical substances which looked promising on the score of chemical structure were likewise tested and some of them give interesting results but none compares with tryptophane.

At this point it looked as though tryptophane was the sole determining factor for bile salt production in the body (dog) but this illusion was promptly dispelled by subsequent experiments with sugar plus tryptophane feeding which gave practically no bile salt increase.

Evidently some other substance present in gelatin is needed together with the tryptophane to raise the bile salt output to the high meat product level.

### *Experimental Observations.*

The experimental methods have been described in Paper I of this series. The sugar solutions alone or with other chemicals were given by stomach tube in two or three fractions during the daytime. The gelatin and lard mixtures were also administered by stomach tube in two or three fractions during the midday period. It is readily appreciated that dogs on a gelatin diet are difficult clinical problems. They may vomit and spoil the experiment. The greatest care is needed in giving the gelatin to prevent vomiting. The dog will tolerate this diet only a relatively few days and there is a steady loss of weight. It is necessary to interrupt the experiments after a few days of gelatin feeding and put the dog on a liberal salmon-bread diet for a few days to restore the animal to normal weight.

Table 31 shows accurately the behavior of this dog on a gelatin diet of 225 gm. of gelatin plus 50 gm. of lard. The bile salt output level after 3 to 4 days of gelatin feeding falls to 1000 to 1100 mg. or about 70 mg. of bile salt per kilo of body weight per 24 hours. We may assume that this dog like many others shows a bile salt output on salmon-bread of approximately 100 mg. per kilo of body weight per 24 hours. When the dog is changed from the salmon-bread diet to gelatin feeding we note a progressive fall to the gelatin feeding level of bile salt output.

Subsequent periods in which the gelatin-lard diet is supplemented by liberal amounts of tyrosine or *p*-hydroxyphenylglycine show exactly similar bile salt output figures, the last day of the period showing about 1000 mg. of bile salt total output.



*Tryptophane* (5 gm.) added to the gelatin-lard diet in this carefully standardized dog gives entirely different results. Instead of a gradual fall we note a prompt rise to 2730 mg. of bile salt output (180 mg. of bile salt per kilo of body weight per 24 hours) which is sustained and is a figure close to the expected level following meat

TABLE 31.

*Gelatin Combined with Tryptophane, Tyrosine, and p-Hydroxyphenylglycine.*  
Dog 26-162.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total taurocholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
11-20	15.9	Gelatin 225, lard 50				
21	15.1	" 225, " 50	35	1.02	36	1370
22	14.9	" 225, " 50	22.5	1.78	40	1480
23	14.5	" 225, " 50	16.5	1.81	30	1110
24	14.7	Salmon-bread diet.	16.0	1.90	30	1110
29		Gelatin 225, lard 50	118			
30	15.2	Gel. 225, ld. 50 + tyrosine 8	83	0.56	46	1700
12-1	15.0	" 225, " 50 + " 7	56	0.74	41	1510
2	14.7	" 225, " 50 + " 7	25	1.20	30	1110
3	14.3	Salmon-bread diet.	16	1.57	25	917
6	15.8	Gelatin 225, lard 50	116			
7	15.2	Gel. 225, ld. 50 + glycine 7	93	0.44	41	1510
8	14.7	" 225, " 50 + " 7	63	0.58	37	1370
9	14.4	" 225, " 50 + " 7	37	0.96	36	1330
10	14.0	Salmon-bread diet.	17	1.45	25	920
27	15.6	" 440	156	0.44	69	2550
28	15.6	Gelatin 225, lard 50	127			
29	15.2	Gel. 225, ld. 50 + tryptophane 5	39	0.79	31	1140
30	15.0	" 225, " 50 + " 5	86	0.66	57	2100
31	15.0	" 225, " 50 + " 5	122	0.61	74	2730
1-1	14.9	Salmon-bread diet.	92	0.77	71	2620

24 hour bile collection at 8.00 a.m.

Bile fistula operation November 2. Bile sterile throughout.

products feeding. The high bile salt output of December 27 is explained by the preceding 5 day period of kidney diet (not included in Table 31).

The *bile volume* (Table 31) is likewise interesting. We note that the curve on gelatin feeding shows a progressive fall from day to

day, more so than the actual fall in bile salt output so that the bile salt concentration per cc. of bile increases above 1 mg. of salt per 1 cc. of bile. The same holds for gelatin plus tyrosine or *p*-hydroxyphenylglycine.

Tryptophane added to the gelatin-lard diet gives a large chologogue effect with an output of 100+ cc. of bile per 24 hours.

TABLE 32.

*Tryptophane, Salmon-Bread, and Sugar Feeding.*

Dog 27-291.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
				In 1 cc. bile.	Total out- put.	
	kg.	gm.	cc.	mg.	mg.	mg.
8-6	12.0	Salmon-bread 330	94	0.39	37	1370
7	12.1	" 220	93	0.36	33	1220
8	12.1	" 330 + tryptophane 5	67	0.54	36	1330
9	12.2	" 330 + " 5	181	0.26	47	1740
10	12.5	" 330 + " 5	244	0.20	49	1810
11	12.3	" 330	201	0.26	52	1920
12	12.3	" 330	123	0.40	49	1810
13	12.4	" 330	113	0.41	46	1700
14	12.0	Fasting.	91	0.38	35	1290
15	11.8	Cane sugar 225	39	0.66	26	960
16	11.7	Sugar 225, + tryptophane 5	16	0.95	15	554
17	12.0	" 225, + " 5	108	0.20	22	810
18	11.8	" 225, + " 5	125	0.080	10	367
19		Salmon-bread 165	120	0.089	11	405
20	11.4	" 330	108	0.30	32	1180
21		" 330	98	0.41	40	1480
22	11.6		86	0.49	42	1550

24 hour bile collection made at 8.00 a.m.

Bile fistula operation July 27.

Bile culture positive throughout but animal in excellent condition.

We might suspect this to be due to the bile salt production but Table 32 shows this to be an unsatisfactory explanation.

Table 32 gives additional data on *tryptophane feeding*. This dog shows the usual bile salt output level on standard salmon-bread—100 mg. of bile salt per kilo of body weight per 24 hours.

When tryptophane (5 gm. daily) is added to the salmon-bread diet we note a prompt and sustained rise in bile salt output to about 150 mg. of bile salt per kilo of body weight per 24 hours. This is not as great as in the gelatin-tryptophane experiment (Table 31) but it is distinct and sustained. Moreover there is a "carry over" into the subsequent control salmon-bread diet period.

Fasting and sugar feeding later reduce the bile salt output close to the fasting base-line level. *Tryptophane* given now with sugar is associated with a fall to the fasting level of about 400 mg. of bile salt per 24 hours or about 35 mg. of bile salt per kilo of body weight per 24 hours. We feel that this observation is of great significance, sustained as it is through 3 days of feeding, and indicates that *tryptophane alone* is not sufficient to enable the body to form any exogenous bile salt. The endogenous bile salt fasting output level is reached during this period. After this period the salmon-bread diet causes a prompt and rather unusually high output, so much so that we may suspect some influence of the preceding tryptophane feeding. If some of the tryptophane excess were retained a day or two in the body this slightly increased bile salt output would be adequately explained.

*Bile volume* output is of unusual interest as we have no knowledge of a similar observation. The *cholagogue action* of tryptophane plus salmon-bread is very well marked—more so than in the gelatin experiments (Table 31) and reaches a total volume of over 240 cc. This volume falls during the subsequent salmon-bread period and more so in the fasting and sugar period. Tryptophane plus sugar gives a notable cholagogue effect—over 100 cc. in spite of no bile salt increase. This is quite contrary to expectations and indicates some substance other than bile salts in this bile which gives a cholagogue effect. One might suspect that tryptophane might be excreted in the bile as a result of its ingestion in 5 gm. doses but the bile analysis gives no indication that any surplus of amino nitrogen-containing substance is present in the unhydrolyzed fraction during such periods. This would exclude the presence of tryptophane or other uncombined amino acids as being responsible for this cholagogue effect.

Table 33 is a continuation of the observations tabulated in Table 31. There was an interval of over a month and the bile is no longer sterile. The dog is in good clinical condition and the

bile flow regular except on the next to the last day (February 17) when an unexplained drop in bile output is noted followed by a compensatory increase on the last day—probably a partial obstruction due to mucus or sediment in the fistula tubing.

These two chemicals—*isatin* and *indigo*—structurally seemed close enough to cholic acid and tryptophane to warrant a physiological test. The accepted structural formulas are given below. The gelatin-lard diet level of about 1000 mg. output per 24 hours is exceeded in all these periods where isatin and indigo are added.

TABLE 23.

*Isatin and Indigo to Supplement Gelatin and Lard Diet.*

Dog 26-162.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
				In 1 cc. bile.	Total out- put.	
	kg.	gm.	cc.	mg.	mg.	mg.
2-6	15.9	Gelatin 225, lard 50	99			
7	15.6	" 225, " 50 + isatin 3.5	104	0.26	27	995
8	15.0	" 225, " 50 + " 3.5	117	0.30	36	1370
9	14.8	" 225, " 50 + " 3.5	148	0.34	50	1850
10	14.9	Salmon-bread diet.	159	0.40	64	2360
15	14.8	" 225, lard 50 + indigo 3.5	89	0.44	39	1440
16	14.4	" 225, " 50 + " 3.5	74	0.56	41	1510
17	14.0	" 225, " 50 + " 3.5	30	0.55	17	625
18	13.7	Salmon-bread diet.	67	0.50	34	1250

24 hour bile collection made at 8.00 a.m.

Bile fistula operation November 2. Bile sterile until about January 13.

Dog on bread diet for several days prior to February 6.

This by itself is of considerable interest but subsequent experiments are negative with isatin (Table 34). We feel that more observations should be obtained dealing with isatin and indigo alone and combined with other chemical factors and various diets. It is not safe to dismiss these two substances as totally inert in bile salt metabolism.

A cholagogue effect is conspicuous with the isatin feeding and distinct with the indigo feeding. These experiments should be compared with Table 34. The feces have a green color during indigo diet periods, indicating incomplete absorption.

Table 34 is of interest because the first experiment (Dog 26-136) is quite decisive. In this dog at least the isatin has no positive effect on the bile salt output. There is a uniform control fore period on gelatin-lard diet. Subsequent *isatin* feeding plus gelatin-lard shows a distinct *decrease* in bile salt output which is quite different from the reaction noted in Table 33.

TABLE 34.

*Gelatin and Lard Diet Supplemented With Isatin.*

## Dog 26-136.

Date.	Wt.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
				In 1 cc. bile.	Total output.	
	kg.	gm.	cc.	mg.	mg.	mg.
3-26		Gelatin 225, lard 50				
27	13.3	" 225, " 50	45	0.69	31	1140
28	13.0	" 225, " 50	24	0.99	24	885
29	12.8	" 225, " 50	18	1.68	30	1105
30	12.7		15	2.10	31	1140
4-3	14.3	Gelatin 225, lard 50 + isatin 5	58			
4	13.2	" 225, " 50 + " 5	34	0.49	17	625
5	12.4	" 225, " 50 + " 5	86	0.35	30	1105
6	12.4	" 225, " 50 + " 5	65	0.34	22	810
7			47	0.55	26	960

*Cane Sugar and Isatin.*

## Dog 27-188.

5-10	13.1	Sugar 300 + isatin 4	103	0.29	30	1105
11	12.5	" 300 + " 4	22	0.47	10.3	380
12	12.4	" 300 + " 4	146	0.088	12.9	475
13	12.1	" 300 + " 4	127	0.134	17.0	625

24 hour collections made at 8.00 a.m.

Dog 26-136, bile fistula operation March 13. Bile culture first positive April 1.

Dog 27-188, bile fistula operation April 25. Bile sterile throughout.

The second experiment (Dog 27-188) likewise shows isatin as inert when added to a sugar diet. It is true that the absolute sugar base-line is not attained in the last 2 days of this experiment but the increase is trivial and would indicate very little potency in the isatin.

Cholagogue effect however is distinct in all these experiments with isatin and in this respect it resembles tryptophane (Table 32). Evidently some substance (intermediate or derivative) has a stimulating effect upon the liver—presumably upon the hepatic epithelium. It is of interest that this effect should be dissociated from the bile salt effect. At least this suggests close kinship between the bile salts, isatin, and indigo.

TABLE 35.  
*Isatin and Salmon-Bread Feeding.*

Dog 27-146.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
			In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	mg.
5-3	Salmon-bread 440	144			
4	" 440 + isatin 4	70			
5	" 440 + " 4	275	0.18	49	1810
6	" 310 + " 4	315	0.18	57	2100
7	" 440 + " 4	290	0.19	55	2030
8	" 440 + " 4	360	0.15	54	1990
9	" 440	345	0.16	55	2030
10	" 440	222	0.22	49	1810
11	" 440	127	0.33	42	1550
12	" 200	164	0.29	48	1770

24 hour collection made at 8.00 a.m.

Bile fistula operation March 2. Bile culture positive throughout but dog in good clinical condition.

Weight during experimental period 13.7 to 13.1 kilos.

Isatin fed by stomach tube shortly after the dog's food had been eaten.

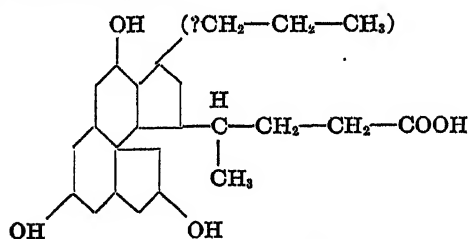
Table 35 should be compared with Tables 33 and 34 which give irregular results with gelatin feeding plus isatin. One dog gives negative and another positive results. This experiment (Table 35) gives a suggestion that isatin added to the standard salmon-bread causes a slight rise in bile salt output. The average salmon-bread level of 1600 mg. per 24 hours is to be compared with the isatin level of 2000 mg. per 24 hours. Evidently more experiments are needed to give a more complete understanding of the influence of isatin upon bile salt metabolism.

We have some experiments with taurine feeding which confirm

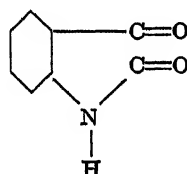
those of Foster, Hooper, and Whipple (2) and show that taurine alone exerts no influence upon bile salt output in bile fistula dogs. We have made observations on the taurine fraction and the cholic acid fraction both obtained from hydrolysis of taurocholic acid. The taurine fraction is inert and the cholic acid fraction is potent, indicating that the cholic acid radicle is the essential factor in bile salt metabolism. These experiments will be reported subsequently.

## DISCUSSION.

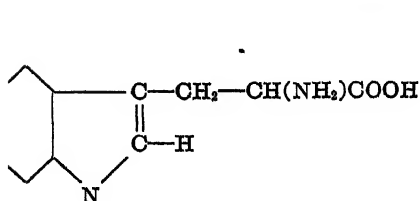
For convenience in this discussion we submit the accepted formulas of cholic acid, tryptophane, isatin, *p*-hydroxyphenylglycine, and indigo.



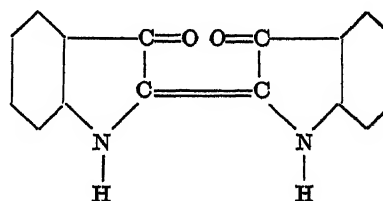
Cholic acid (approximate formula).



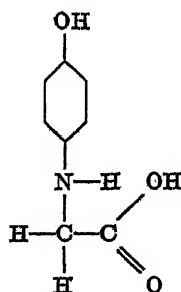
Isatin.



Tryptophane.



Indigo.



Foster, Hooper, and Whipple (2) have given adequate discussions of the reasons why cholic acid is the limiting factor in bile salt metabolism. At all times under normal circumstances there is available an excess of taurine in the body for linkage with any new formed cholic acid to yield taurocholic acid. It seems probable that cystine is the source of most of the taurine.

*Cholic acid* therefore is the substance on which we must focus our attention. A careful and laborious study of its structural formula has been carried on recently by Wieland and his coworkers (4, 5). The formula above represents their general conclusions. Apparently they are satisfied that there are two indene rings (combined 6-carbon and 5-carbon ring). The position of the hydroxyl groups may be somewhat uncertain. The position of the 3-carbon group placed in parenthesis with question mark they leave open, to be determined by subsequent work.

From our standpoint the 5- and 6-carbon indene ring carries the main burden of interest. The indole ring of tryptophane and isatin appears to have some physiological relationship although it is difficult to postulate a replacement of the nitrogen in the indole ring by carbon to form the indene ring. But it does look as though the body utilized in some way the indole ring to build up the cholic acid with its two indene rings. It is of interest that tyrosine and *p*-hydroxyphenylglycine with their 6-carbon rings do not contribute to the production of bile salt, although these substances combined with others might do so.

The cholagogue action deserves critical consideration. We note a distinct cholagogue effect due to tryptophane, isatin, and even indigo. This occurs in the face of heavy sugar feeding which alone always brings about extreme bile concentration. It is possible that this means a direct stimulus to the liver cells, dissociated from the common stimulus of bile salts. This evidence speaks for some kinship between the bile salts, tryptophane, isatin, and even indigo.

#### SUMMARY.

Gelatin feeding in bile fistula dogs yields a very low bile salt output—about 70 mg. of bile salt per kilo per 24 hours. This is to be compared with the standard salmon-bread output level of about 100 mg. of bile salt per kilo of body weight per 24 hours.



Supplementing the gelatin feeding with tyrosine gives negative results.

Tryptophane added to the gelatin diet will give a sustained rise to approximately a low meat diet level or 180 mg. of bile salt per kilo of body weight per 24 hours.

Isatin and indigo may also influence the output of bile salts but this reaction is not constant and less marked when present than noted for tryptophane.

*p*-Hydroxyphenylglycine like tyrosine is inert.

A cholagogue effect is conspicuous when tryptophane, isatin, or indigo is added to the diet, even combined with sugar feeding.

There may be complete dissociation of this cholagogue effect and the bile salt effect.

Tryptophane added to a sugar diet is inert except for a notable cholagogue effect. There is no increase in the bile salt output.

Tryptophane evidently is an important determining factor in bile salt metabolism. Some other substance or substances present in gelatin are needed together with tryptophane to complete the cycle of bile salt production.

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## BILE SALT METABOLISM.

### IV. HOW MUCH BILE SALT CIRCULATES IN THE BODY?

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(Received for publication, October 1, 1928.)

It is generally accepted that bile salts have an "internal circulation;" that is, bile salts are secreted through the bile into the intestine where these salts are promptly absorbed and resecreted into the bile through the hepatic epithelium. It is recognized that the bile salts in the intestinal portion of this cycle have important functions in digestion and absorption of food substances—for example the fats. It is possible that the intrahepatic portion of this cycle is equally important for the internal metabolism of the hepatic cells which are obviously concerned with many vital processes. When we inquire what is known of this intrahepatic sector of this cycle of bile salts complete silence ensues.

Comment has been made elsewhere (4) about the familiar statement that bile salt absorption and elimination in the bile is quantitative. As tested in simple experiments, within limits this is true but there is a constant endogenous production of bile salts (Paper I) as well as the exogenous (food) production, which together with quantitative absorption and bile secretion would lead eventually to infinity. Obviously there is a regulatory mechanism, but in the normal dog how much bile salt is involved in this "internal circulation" and what becomes of the surplus amounts of bile salt? Does the normal hepatic exchange keep in circulation 1 gm. or 1 kilo of bile salts to meet the varying needs of the body?

The ideal experiment is yet to be done. This might be possible with the double return fistula of McMaster and Elman (2), whereby bile may be sampled or collected quantitatively or shunted back into the duodenum. The experiments below probably come close to the truth as regards the actual total of bile salts concerned in

this "internal bile salt circulation" as it goes on from day to day. In our experiments the total bile collection for 24 hours was given back. Probably the passage through the stomach introduces no difficulties but the large doses of bile salts in whole bile may have a somewhat different effect than does normal continuous bile salt flow into the duodenum of the normal dog. We believe our figures if anything are rather lower than would be the figures obtained by sampling the normal flow while the regular continuous bile flow into the duodenum was kept up.

It is apparent from the experiments tabulated below that the dog has a considerable mass of bile salt in circulation when the secreted bile is continuously fed back to the animal. This may amount to 8 or 9 gm. of bile salt as measured by the 24 hour secretion during total refeeding of bile.

In all experiments we note the usual level of bile salt output on the standard salmon-bread diet— $100 \pm$  mg. of bile salt per kilo of body weight per 24 hours. When the whole bile output for each 24 hours is given daily by stomach tube we note a rising curve of bile salt output (Chart A). This goes on steadily in all the experiments up to a level of approximately 700 mg. of bile salt per kilo of body weight per 24 hours. It takes several days to reach this plateau-like level but there is no increase much above this level in the various dogs studied. If supplementary bile is added to the secreted bile and fed with it the dog will show an even higher level but this level will not be sustained when the supplementary feeding is discontinued. We note a prompt fall back to the plateau level when supplementary feeding is stopped (Table 43).

When very large doses of bile are given we note that only a part will be eliminated in the bile (Table 44). Where does this excess of bile salt escape or is it stored or broken down in the body? It is possible that a little of the bile salts may appear in the urine during these excess feeding experiments as we know that small amounts (200 to 300 mg.) of bile salt may appear in the urine in periods of icterus—there is more elimination early than later after the bile obstruction is established (1). After large quantities of bile are fed the feces are quite dark and there is some diarrhea. It is possible that some bile salts together with bile pigments are eliminated with the feces, but we must recall the great power of the intestine to absorb bile salts. There are great difficulties to overcome before we

can measure quantitatively any bile salts which might be found in the feces. Schmidt and Clark (3) show that with a moderate dose of bile salt (7 gm.) by mouth no evidence of its secretion is observed in the urine.

TABLE 41.  
*Refeeding of Bile. Salmon-Bread Diet.*

Dog 26-97.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
			In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	mg.
3-12	Salmon-bread 275	40	1.07	43	1530
13	" 220	46	0.87	40	1470
14	" 440	68	0.43	29	1065
15	" 297	58	0.61	35	1290
16	" 187 + 78 cc. bile.	78	0.86	67	2460
17	" 440 + 73 "	73	0.59	43	1530
18	" 231 + 181 "	181	0.38	69	2540
19	" 231 + 153 "	153	0.54	83	3050
20	" 308 + 200 "	200	0.54	108	3960
21	" 440 + 168 "	168	0.75	126	4640
22	" 286 + 196 "	196	0.78	153	5620
23	" 165 + 223 "	223	0.71	158	5810
24	" 303 + 209 "	207	0.73	151	5550
25	" 231 + 218 "	218	0.89	194	7150
26	" 231 + 167 "	167	0.86	144	5300
27	" 371 + 215 "	215	0.97	206	7560
28	" 242 + 245 "	245			
29	" 264 + 237 "	237	0.93	221	8130
30	" 303 + 220 "	220	1.07	236	8700
31	" 95 + 270 "	270	0.88	236	8700
4-1	" 215 + 191 "	191	1.12	214	7850

24 hour bile collection made at 8.00 a.m.

Bile fistula operation January 7. Bile sterile throughout.

Weight during experimental period 10.5 to 11.4 kilos.

### *Experimental Observations.*

All methods are described in Paper I. All the dogs were on a standard salmon-bread mixture fed daily as indicated in the tables. The bile collections during bile feeding were usually multiple because of the very large volume secretion but the total bile was given

by stomach tube twice daily. Great care must be used to prevent vomiting and the dog must be watched carefully to guard against regurgitation and loss of some bile during the hour or two following the feeding by stomach tube.

Table 41 shows a typical experiment with total refeeding of all secreted bile from day to day. We note a gradual increase from the

TABLE 42.  
*Refeeding of Bile. Salmon-Bread Diet.*

Dog 26-61.

Date.	Diet.	Vol. of bile in 24 hrs.	Amino nitrogen.		Total tauro- cholic acid.
			In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	mg.
11-22	Salmon-bread 462	184	0.227	42	1550
23	" 495 + 190 cc. bile.	199	0.195	39	1440
24	" 440 + 270 " "	270	0.218	59	2180
25	" 550 + 175 " "	175	0.40	70	2580
26	" 550 + 275 " "	275	0.39	108	3990
27	" 550 + 308 " "	308	0.32	99	3650
28	" 550 + 375 " "	375	0.44	165	6100
29	" 396 + 425 " "	425	0.44	187	6900
30	" 292 + 365 " "	390	0.55	215	7950
12-1	" 220 + 500 " "	550	0.37	204	7550
2	" 440 + 475 " "	475	0.35	167	6170
3	" 495 + 460 " "	460	0.45	208	7700
4	" 550 + 420 " "	420	0.52	220	8130
5	" 495 + 500 " "	500			
6		450	0.50	225	8320

24 hour bile collection made at 8.00 a.m.

Bile fistula operation October 29. Bile culture first positive November 30.

Weight during experimental period 13.7 kilos.

standard salmon-bread bile fistula level (bile exclusion) of about 1100 mg. of bile salt per 24 hours. 2 weeks of refeeding are required to raise the level to a maximum of 8700 mg. of bile salt per 24 hours or almost 800 mg. of bile salt per kilo of body weight per 24 hours. The only way to raise the bile salt output above this plateau is to add large amounts of whole bile or bile salt to the usual bile refeeding (Table 43).

TABLE 43.

*Bile Refeeding and Supplementary Bile Feeding.*

Dog 27-256.

Date.	Diet.	Bile fed.			Bile secreted.			Total tauro- cholic acid.
		Vol.	Total amino N.	Total tauro- cholic acid.	Vol.	Amino nitrogen.		
						In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	cc.	mg.	mg.	
6-14	Salmon-bread	264	0	0	124	0.27	34	1,260
15	"	330	0	0	72	0.35	25	925
16	"	253	0	0	152	0.28	43	1,590
17	"	330	0	0	122	0.34	41	1,520
18	"	297	0	0	147	0.28	41	1,520
19	"	109	162		162			
20	"	330	242		242			
21	"	330	269	137	269	0.51	137	5,050
22	"	330	258	180	258	0.70	180	6,650
23	"	264	266	202	7,450	0.76	202	7,450
24	"	187	263	229	8,430	0.87	229	8,450
25	"	143	756	360	13,300	0.88	243	9,000
26	"	330	740	385	14,200	1.02	265	9,800
27	"	269	393	360	13,300	0.92	360	13,300
28	"	229	336	302	11,100	0.90	302	11,150
29	"	292	331	307	11,300	0.93	307	11,350
30	"	182	800	414	15,250	0.94	290	10,750
7-1	"	330	770	384	14,100	0.96	260	9,600
2	"	231	934	520	19,200	0.92	400	14,800
3	"	330	437	440	16,200	1.01	440	16,250
4	"	330	444	425	15,600	0.96	425	15,700
5	"	330	459	415	15,250	0.91	415	15,300
6	"	330	429	380	14,000	0.88	380	14,000
7	"	330	871	503	18,500	0.90	405	14,000
8	"	330	510	470	17,300	0.92	470	17,400
9	"	330	402	350	12,900	0.87	350	12,900
10	"	330	258	183	6,730	0.71	183	6,750
11	"	330	301	208	7,650	0.69	209	7,670
12	"	330	315	201	7,400	0.64	201	7,410
13	"	330	0	0	322	0.84	271	10,000
14	"	330	0	0	137	0.33	45	1,660

Bile fistula operation June 6. Bile culture positive since June 25.

Weight during experimental period 11.9 to 13.3 kilos.

Dog in excellent clinical condition.

We observe a maximal cholagogue effect within a few days after beginning bile refeeding. After this there is a gradual increase in bile salt concentration per 1 cc. of bile to account for the higher bile salt output.

Table 42 is almost exactly identical with the preceding experiment (Table 41) but this was done before we realized the long period usually required for bile refeeding to effect its maximum

CHART A BILE RE-FEEDING AND SUPPLEMENTARY BILE FEEDING

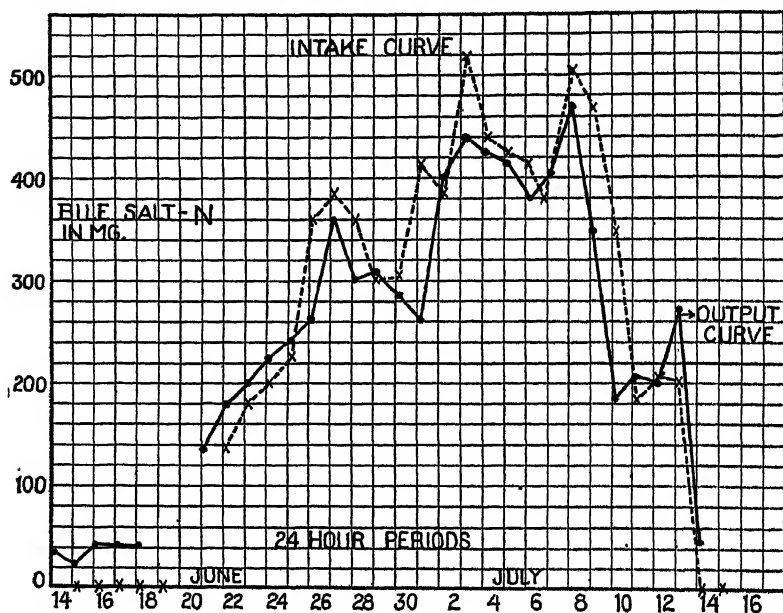


CHART A.

plateau level. It is possible that this dog if continued on the diet a few days longer would have exceeded the recorded maximum.

The initial bile salt output level on salmon-bread with bile exclusion is about 1500 mg. per 24 hours. With daily total bile refeeding we observe a steady rise to 8300 mg. per 24 hours or about 600 mg. of bile salt per kilo of body weight per 24 hours. This maximum figure compares with the results in Table 43 but is distinctly lower than the maximum in Table 41.



The maximum cholagogue effect is noted after 1 week of bile re-feeding. The total figures for this dog are much above those noted in Table 41—in fact, the volume is almost exactly double. This dog was only a little heavier, 13.7 kilos compared with 11.0 kilos. We cannot explain this type of individual difference.

Bile cultures in both experiments (Tables 41 and 42) were negative at first and became positive during the periods of great cholagogue activity, probably in part due to the frequent collection necessitated by the very large volume flow to insure against a full bag and consequent obstruction with back pressure in the biliary tree.

Table 43 gives more experimental data related to bile refeeding. The salmon-bread bile exclusion level is about 1500 mg. of bile salt per 24 hours. Bile refeeding daily of total bile output for 7 days raises the output level to about 9000 mg. of bile salt for 24 hours or about 700 mg. per kilo of body weight per 24 hours.

*Large supplementary feeding* of bile added to the refeeding of total bile output now raises the bile salt to much higher levels, even to 15,000 to 17,000 mg. of bile salt per 24 hours. But we note that the total output of bile salts falls consistently behind the total intake. It is obvious that the supplementary feeding raises the level but bile salt is lost somewhere and not all these large amounts subsequently appear in the bile. Evidently we are reaching the maximum capacity of the liver cell or intestinal tract to handle these bile salts.

When supplementary feeding is discontinued but refeeding carried along as usual we note a drop back rather promptly to the former refeeding plateau or even a little below, to 7500 mg. of bile salt per 24 hrs.

Chart A shows graphically the results given in Table 43. The table gives the events exactly at the time of occurrence. The bile feeding of course influences the output of the next day's collection. On the chart the values for bile salt feeding and secretion are run along so that the curves may be compared more easily—the output and intake values which are related are charted on the same day. This in effect moves back 24 hours the secretion curve.

In Table 43 we do not give the amounts of *supplementary feeding* in actual figures but these can be obtained readily as the difference between the bile secreted and bile refed on any given day. Chart

A shows the curve of intake constantly exceeding the output level during supplementary bile feeding periods.

TABLE 44.  
*Heavy Supplementary Bile Feeding.*

Dog 27-256.

Date.	Diet.	Bile fed.			Bile secreted.			Total tauro- cholic acid output.
		Vol.	Total amino N.	Total tauro- cholic acid.	Vol.	Amino nitrogen.		
						In 1 cc. bile.	Total output.	
	gm.	cc.	mg.	mg.	cc.	mg.	mg.	mg.
7-15	Salmon-bread 330	0	0	0	150	0.26	39	1,440
16	" 330	0	0	0	114	0.26	29.5	1,090
17	" 330	0	0	0	74	0.26	19.3	714
18	" 260	0	0	0	183	0.28	51	1,880
19	" 260	0	0	0	150	0.30	45	1,660
20	" 187	600	195	7,150	144	0.26	37.3	1,375
21	" 330	0	0	0	282	0.75	211	7,743
22	" 330	0	0	0	148	0.27	40	1,475
23	" 330	0	0	0	94	0.30	28.2	1,040
24	" 330	0	0	0	145	0.27	39	1,440
25	" 330	0	0	0	173	0.29	50	1,850
26	" 330	200	78	2,860	127	0.32	41	1,500
27	" 330	0	0	0	224	0.61	137	5,020
28	" 330	0	0	0	147	0.30	44	1,625
29	" 330	0	0	0	82	0.34	27.9	1,030
30	" 330	0	0	0	160	0.35	56	2,070
31	" 220	0	0	0	157	0.32	50	1,730
8-1	" 260	0	0	0	125	0.38	48	1,770
2	" 330	0	0	0	145	0.33	48	1,770
3	" 330	0	0	0	125	0.38	48	1,770
4	" 165	1000	570	21,000	134	0.33	45	1,650
5	" 330	0	0	0	372	0.97	360	13,200
6	" 330	0	0	0	123	0.27	33	1,220
7	" 330	0	0	0	148	0.26	39	1,440

Bile fistula operation June 6. Bile culture throughout experimental period.

Clinical condition good.

Weight during experimental period 12.7 to 13.3 kilos.

Table 44 shows the influence of large doses of bile salt given by mouth to standard bile fistula dogs on the salmon-bread diet. The results are also given in Chart B. These large amounts of bile salt

were given as thick syrupy dog bile concentrated over the water bath to one-fourth its original volume but the figures given in the table refer to the original bile before it was so concentrated.

Evidently large doses of bile salt (21 gm.) by mouth are not accurately reflected in the output values (13 gm.) as observed in the

CHART B BILE RE-FEEDING

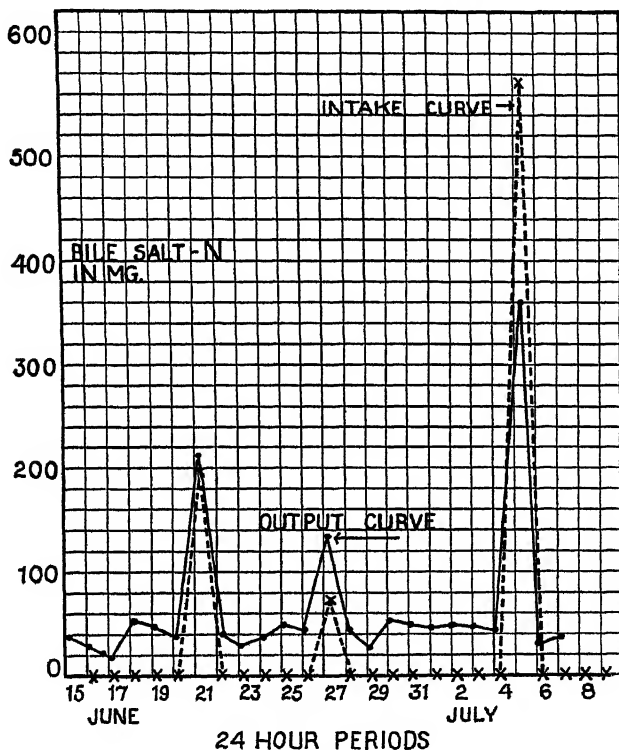


CHART B.

bile fistula secretion. As to what becomes of these many grams of excess bile salts we cannot venture an opinion.

Table 45 gives the bile salt secretion during 2 to 16 hour periods, following the heavy bile feeding (7 to 21 gm. of bile salt). The speed of this bile salt circulation has often been noted. With

small doses the salt may be all eliminated within 8 to 10 hours. With larger doses it takes somewhat longer but even with the maxi-

TABLE 45.

*Heavy Supplementary Bile Feeding. Bile Collection at Short Intervals.*  
Dog 27-256.

Date.	Diet.	Bile fed.			Hrs. of secretion.	Bile secreted.			Taurocholic acid.	
		Vol.	Total amino N.	Total taurocholic acid.		Vol.	Amino nitrogen.		Total	Per hr.
							In 1 cc. bile.	Total out-put.		
	gm.	cc.	mg.	mg.		cc.	mg.	mg.	mg.	mg.
7-19	Salmon-bread 253 9.00 a.m.	0	0	0	24	150	0.30	45	1660	69
20	Salmon-bread 187 8.40 a.m. 4.30 p.m.	600	195	7,150	24 8	144 99	0.26 0.86	37.3 85	1375 3120	57 390
21	Salmon-bread 330 8.25 a.m.	0	0	0	16	183	0.69	126	4630	290
22	Salmon-bread 330 9.10 a.m.	0	0	0	25	148	0.27	40	1475	59
8-3	Salmon-bread 200	0	0	0						
4	Salmon-bread 330 8.45 a.m. 2.45 p.m. 4.55 " 11.50 "	1000	570	21,000	24 6 2.1 7	134 130 70 127	0.33 1.16 1.14 0.88	45 151 80 112	1650 5550 2950 4110	69 925 1405 587
5	Salmon-bread 330 8.45 a.m.	0	0	0	9	45	0.39	17.6	645	72
6	Salmon-bread 330 8.20 a.m.	0	0	0	23.5	123	0.27	33	1210	53

All bile feeding by stomach tube at 8.45 a.m. and 3.00 p.m.

Weight during experimental period 12.7 to 13.3 kilos.

mum doses (21 gm.) the bile salt is largely eliminated within 15 hours. The second 24 hours show no residue of bile salt, in fact in

some instances the figures are below the average during the second 24 hours following heavy bile feeding.

#### SUMMARY.

Daily bile refeeding of all bile secreted from the bile fistula will raise the bile salt secretion to a fairly stable plateau level of 700 to 800 mg. of bile salt per kilo of body weight per 24 hours.

According to these experiments a 10 kilo dog normally keeps in circulation about 7 or 8 gm. of bile salt during each 24 hours.

Ingestion of large amounts of bile salts results in a very prompt resecretion in the bile fistula dog. This excess is almost wholly eliminated during the next 8 to 16 hours.

Supplementary feeding may raise the level of bile salt secretion to very high levels—even 15 to 17 gm. per 24 hours—but the output always falls behind the intake. The difference is most notable with the largest feedings of bile salts. The body evidently can handle or turn over only about so much bile salt and the surplus is lost. The fate of this surplus is a mystery.

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## THE BIURET REACTION.

### I. THE BIURET REACTION OF ACID IMIDES OF THE BARBITURIC ACID TYPE.\*

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The biuret reaction is the color reaction shown by biuret,  $\text{NH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{NH}_2$ , malonamide,  $\text{NH}_2\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}_2$ , oxamide,  $\text{NH}_2\cdot\text{CO}\cdot\text{CO}\cdot\text{NH}_2$ , and many related compounds when treated with cupric salts and alkali. The discovery by Ritthausen<sup>1</sup> that proteins behave in a similar fashion with the same reagents led to the practical application of the biuret reaction, and it has been in common use as a sensitive test for proteins for some 50 years.

The reaction was first observed by Wiedemann<sup>2</sup> with biuret itself. Schiff<sup>3</sup> did the pioneer work upon the chemistry of the biuret reaction: he isolated the product of the reaction of biuret itself with cupric acetate and potassium hydroxide,<sup>4</sup> established, by means of analyses, a formula for the salt,  $\text{K}_2\text{Cu}(\text{biuret})_2$ , and proposed a structure. Recent evidence that other di-acid amides which show the biuret reaction form products having the formula  $\text{Me}_2\text{Cu}(\text{amide})_2$ , just as does biuret, itself a di-acid amide, has been obtained in the study made by Rising, Hicks, and Moerke<sup>5</sup> of the

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\* The contents of this paper were reported at the Midwest Regional Meeting of the American Chemical Society held at Minneapolis, June, 1928.

† The work on the alkali copper salts of diethylbarbituric acid herein described was done by C. A. Johnson in partial fulfilment of the requirements for the degree of Master of Science at the University of Chicago, 1927. The experimental work was carried out at the University of Illinois Medical School.

<sup>1</sup> Ritthausen, H., *J. prakt. Chem.*, 1873, vii, 361.

<sup>2</sup> Wiedemann, G., *Ann. Chem.*, 1848, lxxviii, 323; *J. prakt. Chem.*, 1847, xlii, 255; 1848, xliii, 271.

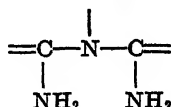
<sup>3</sup> Schiff, H., *Ber. chem. Ges.*, 1896, xxix, 298; *Ann. Chem.*, 1898, ccxcix, 286; 1901, cccxix, 287; 1907, ccclii, 78.

<sup>4</sup> Ley, H., and Werner, F., *Ber. chem. Ges.*, 1913, xlii, 4042, have since isolated another product of the reaction of biuret with alkali and copper salts, having the formula  $\text{KCu}(\text{biuret})\cdot 3\text{H}_2\text{O}$ .

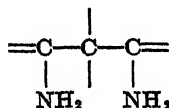
<sup>5</sup> Unpublished data.

nature of the products of the biuret reaction with alkylated oxamides, and malonamide. These di-acid amides behave like biuret in that the products of their reaction with the biuret reagents agree in composition with the formula  $\text{Me}_2\text{Cu}(\text{amide})_2$ .

Schiff also discovered that many compounds structurally related to biuret show an analogous color reaction with copper salts and alkali, but he did not isolate the colored products. He tested chiefly di-acid amides and amino acid amides, and divided those showing a positive reaction into three classes: (1) compounds resembling biuret, with the group



(2) those resembling malonamide, with the group



and (3) those of the oxamide type having the two  $\text{—C=}$  groups directly  
 $\text{NH}_2$

adjoined. Schiff found that two of the carbon valences in the group  $\text{—C=}$  may hold  $\text{O=}$ ,  $\text{S=}$ , or  $\text{HN=}$ .



In his summary of the groups essential for the occurrence of the biuret reaction, Schiff made no provision for the fact that many acid imides, with the characteristic  $\text{—C—N—C—}$  group, show the reaction. Succinimide,



$\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{CH}_2$ , and phthalimide,  $\text{C}_6\text{H}_4\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}$ , for example,

form colored products with the biuret reagents. The first extensive investigation of the biuret reaction of acid imides was made by Tschugaeff,<sup>6</sup> who isolated the products of the reaction of succinimide with cupric salts and the alkali hydroxides. Analyses of these compounds proved that their molecules contain a ratio of 4 molecules of imide to 2 atoms of alkali metal to 1 atom of copper. Ley and Werner<sup>7</sup> confirmed and supplemented

<sup>6</sup> Tschugaeff, L., *Ber. chem. Ges.*, 1905, xxxviii, 2899; 1906, xxxix, 3190; 1907, xl, 1973.

<sup>7</sup> Ley, H., and Werner, F., *Ber. chem. Ges.*, 1905, xxxviii, 2199; 1907, xl, 705.



Tschugaëff's results in a study of the potassium copper derivatives of succinimide and phthalimide. The results obtained by these men justify the choice of the formula  $\text{Me}_2\text{Cu}(\text{imide})_2 \cdot x\text{H}_2\text{O}$  for the products of the biuret reaction of acid imides.

Kober<sup>\*</sup> has proposed certain theories concerning the nature and constitution of products of the biuret reaction of amino acid derivatives and peptides. His ideas are based in part upon an interesting spectrographic study of the colored products of the biuret reaction. We are unable to agree with most of his conclusions pertaining to constitution, and it is wholly impossible to follow his reasoning as to the electronic nature of the amino and imino groups in peptide molecules. Some other suggestions of Kober's are perhaps more valuable and will be discussed in a later paper by one of the present authors.

The painstaking study of the chemistry of the biuret reaction made by Schiff, Tschugaëff, and others thus brought to light a considerable number of important facts regarding the nature of the reaction. Building upon the results of the earlier work the present authors have undertaken to amplify present knowledge of the biuret reaction. We are endeavoring (1) to ascertain more exactly than is now known what atoms or groups of atoms take part in biuret reactions; that is, the arrangement of atoms essential for the occurrence of the reaction. As an aid to the accomplishment of (1), it is desired (2) to isolate the pure products of the biuret reaction with simple molecules, such as biuret itself, oxamide, malonamide, some acid imides, and amino acid amides and polypeptides in order to determine their molecular structure from a study of their properties. With the results obtained from the investigations of (1) and (2) it should be possible (3) to gain some information regarding the constitution of the products of the biuret reaction with proteins and, in particular, to use this information to throw light upon the molecular structure of the proteins themselves. This, indeed, is the ultimate and most vital aim of the work, and the preliminary steps suggested have been undertaken largely as an aid to its accomplishment.

We have continued the study of the biuret reaction, enlarging and clarifying the scope of its application by an examination of the behavior of the two acid imides, diethylbarbi-

<sup>\*</sup> Kober, P. A., and Sugiura, K., *J. Biol. Chem.*, 1912-13, xiii, 1; *Am. Chem. J.*, 1912, xlviii, 383. Kober, P. A., and Haw, A. B., *J. Am. Chem. Soc.*, 1916, xxxviii, 457.

uric acid,  $\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{C}(\text{C}_2\text{H}_5)_2$ , and barbituric acid,  $\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{CH}_2$  (the latter said by Schiff *not* to show the biuret reaction), with alkali and copper salts. These imides are related to succinimide and phthalimide in structure and behavior, and they are even more closely related to biuret, itself an acid imide as well as a di-acid amide. Both barbituric acid and its diethyl derivative show the biuret reaction under favorable conditions, and we have isolated the colored salts formed in their reaction with the biuret reagents. These two salts have been analyzed and their properties studied, molecular structures for them have been devised, and theories developed concerning the mechanism

TABLE I.  
*Analytical Data for Sodium Copper Diethylbarbitate.*

Element.	Percentage composition.		Mean value.	Calculated percentage composition for $\text{Na}_2\text{CuC}_{12}\text{H}_{14}\text{O}_{10}\text{N}_8$ (mol. wt. 877.99).
	Sample I.	Sample II.		
			<i>per cent</i>	
Na	5.37	6.17	5.77	5.24
Cu	7.30	7.01	7.15	7.24
C	44.24	43.79	44.01	43.74
H	5.18	5.23	5.20	5.51
N	13.25	12.93	13.09	12.76
O				25.51
				100.00

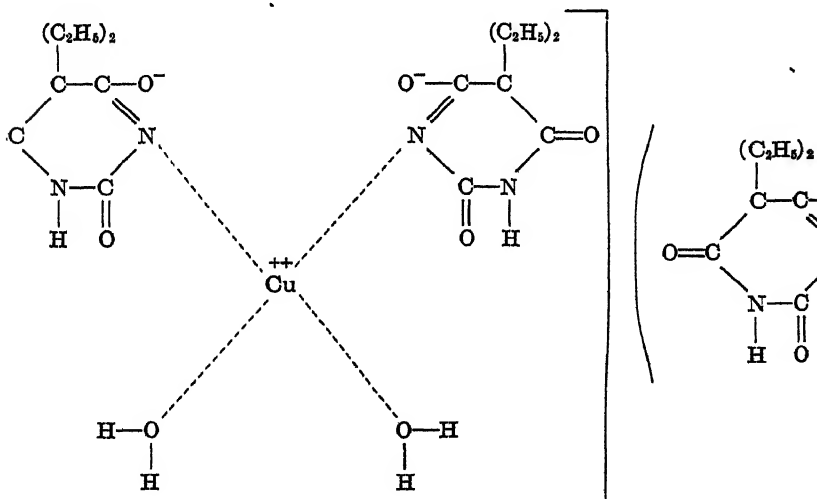
of the reaction in which the salts are formed. The present paper deals with these aspects of the chemistry of sodium copper diethylbarbitate. A later paper will discuss the salt lithium copper barbitate.

Sodium copper diethylbarbitate was prepared by treatment of the sodium salt of diethylbarbituric acid (Merck's "barbital sodium") with cupric acetate in 90 per cent alcohol at room temperature. It was found that no reaction whatever occurs when all water is excluded from the reaction medium, and also that no reaction occurs when more than 10 per cent of water is present. It is to be noted that two deviations from the usual procedure for carrying out the biuret reaction were made: (1) no free alkali was used; (2) only small amounts of water were present in the

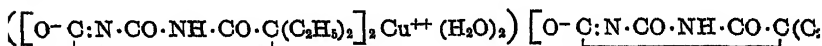
reaction mixture. The probable influence of these conditions upon the course of the reaction will be discussed presently. Sodium copper diethylbarbitate obtained under these conditions is an amorphous substance, lavender in color, characterized by its extreme insolubility in water and other solvents. It is slowly hydrolyzed by water, and is decomposed by carbon dioxide and all acids. The percentage composition of the products of two separate preparations of the salt is shown in Table I. Further details regarding the procedure used to obtain the salt and analyses of the compound are to be found in the experimental part of this paper.

The percentage composition agrees well with that calculated for a molecule of formula  $\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ . On account of the extreme insolubility of the salt in the usual solvents, it has not been possible to carry out molecular weight determinations in solution and it is therefore impossible to state at present whether this empirical formula is also the molecular one. It will be noted that the formula  $\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$  agrees with that previously shown by the work of Tschugaeff and others to be correct for alkali copper salts of acid imides,  $\text{Me}_2\text{Cu}(\text{imide})_4$ ; diethylbarbituric acid is  $\text{C}_8\text{H}_{12}\text{O}_3\text{N}_2$ , and  $\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$  is  $\text{Na}_2\text{Cu}(\text{C}_8\text{H}_{11}\text{O}_3\text{N}_2)_4 \cdot 2\text{H}_2\text{O}$ .

A structure for the salt is proposed:



This structure indicates that sodium hydroxide neutralizes 2 molecules of diethylbarbituric acid; the neutralization enhances the amine character of diethylbarbituric acid, which, reacting as an amine, forms a complex ion of the cupric-ammonium type  $\left( \left[ \text{O}-\text{C}:\text{N}\cdot\text{CO}\cdot\text{NH}\cdot\text{CO}\cdot\text{C}(\text{C}_2\text{H}_5)_2 \right]_2 \text{Cu}^{++} (\text{H}_2\text{O})_2 \right)$ . The copper is very little ionized since it is held in a complex ion, to which the color of the salt must be ascribed. Two auxiliary valences of copper hold amine nitrogen atoms, and two others hold the oxygen atoms of 2 molecules of water. The condensed formula for the salt is

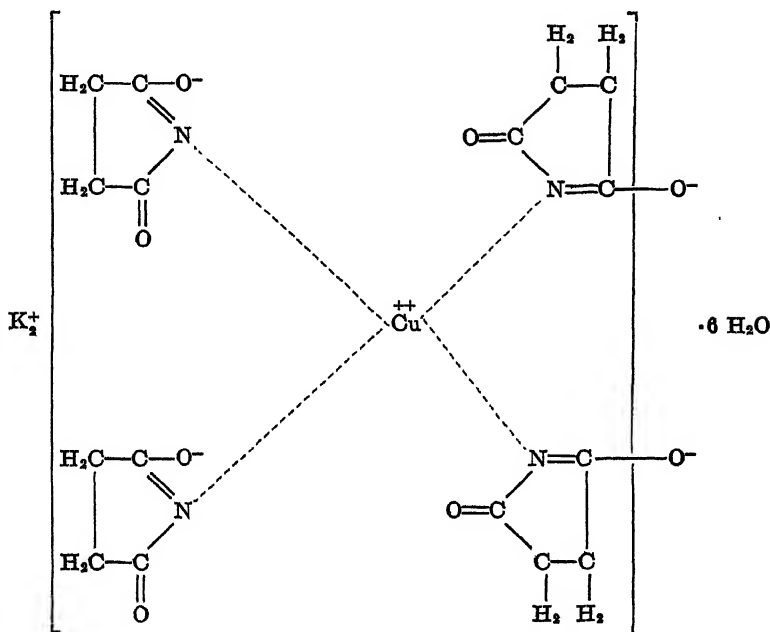


Sodium copper diethylbarbitate would then resemble in structure the well known combinations of amino acids with salts: amino acetic acid,<sup>9</sup> for example, combines with potassium chloride to form the salt  $\text{NH}_2\cdot\text{CH}_2\cdot\text{COOH}, \text{KCl}$ , and with potassium nitrate to form the salt  $\text{NH}_2\cdot\text{CH}_2\cdot\text{COOH}, \text{KNO}_3$ . The structures of these salts must be  $\text{K}^+[-\text{OOC}\cdot\text{CH}_2\cdot\text{N}^+\text{H}_3]\text{Cl}^-$  and  $\text{K}^+[-\text{OOC}\cdot\text{CH}_2\cdot\text{N}^+\text{H}_3]\text{NO}_3^-$ . Here the complex ion is that of nitrogen and hydrogen, as in ammonium ion,  $\text{NH}_4^+$ .

Similar structures are suggested for the alkali copper imides prepared by Tschugaeff and by Ley and Werner. For example, potassium copper succinimide may well have the structure shown in the diagram on the next page. This salt contains 6 molecules of water, as is readily shown by its behavior over sulfuric acid in a vacuum desiccator at room temperature; under these conditions a loss of weight occurs which corresponds exactly to 6 molecules of water. According to theories of structure current at the present time, water so readily removed from a molecule is water of crystallization rather than water of structure and should be placed outside the complex. In the case of sodium diethylbarbitate, however, no loss of weight occurs in a vacuum, nor when the salt is dried in air at  $100^\circ$ . For this reason it is thought best to interpret the analytical data obtained for the latter salt to the effect that its molecule contains 2 molecules of water as water of

<sup>9</sup> Meyer, V., and Jacobson, P. H., *Lehrbuch der organischen Chemie*, Leipsic, 2nd edition, 1913, i, pt. 2, 748.

structure, and to place the water within the complex ion, as is shown in the structure given.



Potassium copper succinimide.

The results of our study of the biuret reaction of acid imides, outlined here and discussed more fully in the experimental part of this paper, have served as a basis for certain conclusions concerning (1) the rôle played by the two "biuret reagents," alkali and cupric ion, in such reactions, (2) the course of the reaction of these reagents with acid imides, and (3) the atoms in the imide molecule which take part in the biuret reaction.

The alkali used for the biuret reaction serves two vital purposes: of prime importance is its behavior as a salt-forming agent which neutralizes the acid imide; moreover, used in excess, the alkali acts to insure the stability of the salt so produced by preventing its hydrolysis, a decomposition to which salts of acid imides are sensitive, as salts of rather weak acids. Let us consider these important functions of the alkali.

Neutralization of the acid amide by the alkali certainly occurs. Succinimide, phthalimide, and diethylbarbituric acid are monobasic acids. We believe that this reaction prepares the way for the formation of a copper complex, in that neutralization does away with the acid properties of the imide and enhances its amine-like character, with the result that it readily enters into copper complexes. It is well known that alkali salts of amino acids, *e.g.*  $[\text{H}_2\text{N}\cdot\text{CH}(\text{R})\cdot\text{COO}^-]\text{Na}^+$ , behave like free amines. In the absence of alkali, barbituric acid is neutralized by copper hydroxide, a green salt,<sup>10</sup>  $\text{Cu}(\text{C}_4\text{H}_3\text{N}_2\text{O}_3)_2\cdot 3\text{H}_2\text{O}$  being formed, and no complex ion. In the presence of alkali, copper hydroxide forms with barbituric acid an alkali copper salt, the copper being in the complex ion. In the absence of alkali, tartaric acid forms a green copper salt,<sup>11</sup>  $\text{CuC}_4\text{H}_4\text{O}_6\cdot 3\text{H}_2\text{O}$ , with copper hydroxide; alkali tartrates react with copper hydroxide to form the well known deep blue alkali copper tartrate in which copper is in a complex ion. Thus when alkali is used to take care of the neutralization of these compounds, formation of the copper complexes readily follows.

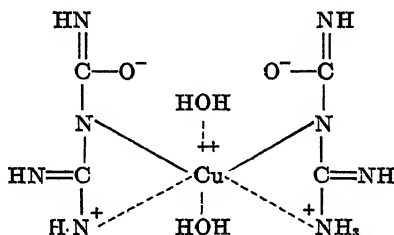
It may be suggested that biuret reactions sometimes occur when no free alkali is present. These cases are undoubtedly significant, but it will be shown that they do not constitute exceptions to the rule that neutralization of the imide and prevention of the hydrolysis of the imide salt must be taken care of to insure the occurrence of a biuret reaction. Biuret reactions which occur without the addition of alkali may be classified as follows:

*Group I.*—Typical members of this group are the iminobiurets, dicyanodiamidine,  $\text{NH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{C}(\text{:NH})\text{NH}_2$ , and biguanide,  $\text{NH}_2\cdot\text{C}(\text{:NH})\text{NH}\cdot\text{C}(\text{:NH})\text{NH}_2$ . These compounds react with copper salts to form deep red products without the addition of any alkali, and the reactions are generally considered to be biuret reactions. The iminobiurets are strongly basic compounds and may themselves replace alkali in their reactions with copper salts; dicyanodiamidine, for example, having marked basic properties, and being at the same time a weak acid like biuret, may neutralize itself, and the internal ammonium salt formed by such "self-neutralization" may then react with copper to form a product

<sup>10</sup> Baeyer, A., *Ann. Chem.*, 1864, cxxx, 142.

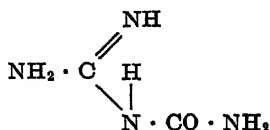
<sup>11</sup> Werther, G., *Ann. Chem.*, 1844, lii, 301.

containing copper in a complex ion. The composition of copper dicyanodiamidine is  $\text{Cu}(\text{N}_4\text{H}_6\text{OC}_2)_2 \cdot 2\text{H}_2\text{O}$ .<sup>12</sup> Its structure may be

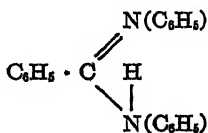


Copper dicyandiamidine.

Here the copper is in an amine complex, and at the same time in a salt of the amidine



Certain amidines are known to be capable of salt formation with metals; *e.g.*, diphenylbenzamidine,



forms a silver salt.

*Group II.*—An example is found in the case of the salt sodium diethylbarbitate, which shows the biuret reaction in 90 per cent alcohol without the addition of free alkali, as has already been described. And indeed no free alkali is needed, since the sodium salt of the imide is already at hand and combines with copper directly to form the complex; this behavior confirms the view already presented that the function of the alkali in the biuret reactions as ordinarily performed is the formation of such an

<sup>12</sup> Grossman, H., and Schück, B., *Ber. chem. Ges.*, 1906, xxxix, 3356.

alkali salt. Free alkali is not needed to prevent hydrolysis, which is obviated by the use of a nearly anhydrous reaction medium.

By reasoning thus, it seemed likely to us that certain substances such as barbituric acid and diethylbarbituric acid, which do not show the biuret reaction under the conditions ordinarily used for the reaction, *i.e.* in the presence of water, would respond to the test in the absence of water. This conclusion has been justified by the results of the study of the behavior of barbituric acid and its diethyl derivative with the biuret reagents in the absence of all but small amounts of water, a behavior overlooked by Schiff, who states that barbituric acid does not show the biuret reaction, and by others, who have studied the behavior of many compounds with the biuret reagents. The explanation for the positive reaction of these compounds when allowed to react under approximately anhydrous conditions is very possibly to be found in the greater stability of their alkali salts in the absence of water. Any conditions, then, which favor the formation and stability of such salts favor the occurrence of a biuret reaction. It might be suggested that the failure of diethylbarbituric acid to show the color reaction in water is due to its extreme insolubility in water. This explanation is untenable since a water solution of the readily soluble sodium salt of the acid also fails to show the reaction.

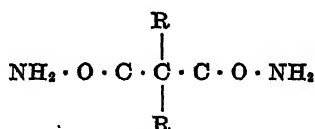
The second biuret reagent, cupric ion, may have as its source either a copper salt or copper hydroxide<sup>13</sup> and has the important function of converting the alkali salts of acid imides into copper derivatives in which the copper is in a complex ion somewhat similar to the well known cupric-ammonium ion. As stated previously, and repeated here for emphasis, the alkali salts of imides are stronger amines than are the imides themselves before neutralization. The enhancement of their amine-like character by salt formation favors in a very decided way the formation of a copper complex, for which a structure has already been suggested.

The mechanism of the reaction of acid imides with cupric ion in alkali is, in all probability, relatively simple. Fundamentally important for the occurrence of the reaction is an acid hydrogen

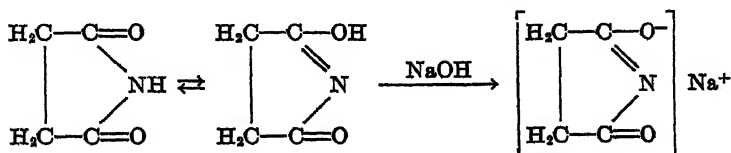
<sup>13</sup> Schiff showed that other metals, such as nickel, form alkali metal salts with biuret and related compounds quite as readily as does copper. We have prepared sodium nickel monoethyl oxamide, which will be discussed in a later paper.



atom in the imide molecule. The salts formed by neutralization of imides are believed to be oxygen, not nitrogen, salts. This being the case, salt formation must be preceded by migration of the replaceable hydrogen atom to oxygen, a tautomeric change undoubtedly aided by alkali; indeed the action of alkali in favoring the formation of acid tautomers of the imides may constitute a third function of alkali as a biuret reagent. We have proved that the hydrogen atom in imide molecules which is essential for the occurrence of a biuret reaction is the one attached originally to nitrogen; N-alkylated succinimides and phthalimides were prepared and the behavior of these substances with the biuret reagents studied. They failed to show the biuret reaction under any conditions used. Hence we have concluded that the hydrogen atoms attached to carbon in such imides have no acid properties, and that the replaceable hydrogen atom of an unsubstituted imide is the one held by nitrogen, and capable of migrating to oxygen under the influence of alkali. Less direct evidence supporting this conclusion is found in the fact that the substituted malonamides of the type



readily show the biuret reaction. Tautomerization and neutralization in the case of succinimide may occur as follows:



The results of the present investigations have furnished some clues as to the identity of the atoms which are essential for the occurrence of the biuret reaction in the case of acid imides. We are convinced that there are 2 atoms in an imide molecule which are intimately concerned in the biuret reaction: (1) a replaceable hydrogen atom, (2) an amine nitrogen atom with a basic character

sufficiently pronounced to insure its ready entrance into a copper complex. That only 1 such acid hydrogen atom and 1 amine nitrogen atom are required is proved by the fact that such imides as succinimide and phthalimide readily show the biuret reaction. Since diethylbarbituric acid behaves with the biuret reagents as do the more typical imides just mentioned, we must conclude that its molecule contains the necessary atoms, and that these are identical with the reactive atoms of succinimide and phthalimide.

One of the present authors (Rising) in collaboration with Dorothy R. Gaston, is continuing the work of the main problem, outlined in the early part of this paper; they are isolating and making a study of the products of the biuret reaction of certain peptides. This part of the biuret research is particularly interesting for its close relationship to the ultimate goal of our work, the discovery of some of the facts regarding protein structure.

Other phases of the biuret problem are being studied at the present time. The establishment of values for molecular weights of products of the biuret reaction is necessary to prove or disprove our theories with regard to structure. For this reason, considerable attention is being devoted to this part of the work, and results will be published in due time.

#### EXPERIMENTAL.

##### 1. *Sodium Copper Diethylbarbitate, $Na_2CuC_{22}H_{48}O_{14}N_8$ .*

Sodium copper diethylbarbitate was obtained by treatment of Merck's barbital sodium with copper acetate in 90 per cent ethyl alcohol. To this end barbital sodium (2 gm.) was dissolved in 300 cc. of 90 per cent alcohol, and there were added to this solution 400 cc. of a saturated solution of copper acetate (0.6832 gm. of  $Cu(Ac)_2 \cdot H_2O$ ) in 95 per cent alcohol. This mixture soon became reddish violet in color, and when it had stood for a short time a lavender solid was precipitated from it. The precipitate, sodium copper diethylbarbitate, was brought upon a filter, washed several times with alcohol, and dried in a vacuum over sulfuric acid. Precautions were used at all times to protect the salt from the moisture of the air and from carbon dioxide. About 1.5 gm. of the salt were obtained from the quantities of reagents stated.

Sodium copper diethylbarbitate is an amorphous substance of

fine lavender color. It is insoluble in water and other ordinary solvents, and is not readily wet by liquids. The salt is, however, slowly hydrolyzed by water, and is decomposed by all acids. It does not melt or decompose up to 300°, and shows no loss in weight in a vacuum over sulfuric acid at room temperature, nor in air up to 100°.

A complete set of analytical data obtained for Sample I (already referred to in the introduction to this paper) follows. The copper content of the salt was determined by electrolysis. Sodium was determined as sodium sulfate in solutions of the salt from which copper had been removed.

0.1880, 0.1963 gm. substance:	0.0139, 0.0143 gm. Cu.
	$\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ . Calculated. Cu 7.24.
	Found. " 7.39, 7.28.
0.1880, 0.1963 gm. substance:	0.0319, 0.0326 gm. $\text{Na}_2\text{SO}_4$ .
	$\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ . Calculated. Na 5.24.
	Found. " 5.49, 5.37.
0.1819, 0.1889 gm. substance:	22.64 cc. $\text{N}_2$ (30°, 749.0 mm.), 23.41 cc. $\text{N}_2$ (31°, 748.6 mm.) (over 50 per cent KOH).
	$\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ . Calculated. N 12.76.
	Found. " 13.31, 13.25.
0.1826, 0.1819 gm. substance:	0.2963, 0.2949 gm. $\text{CO}_2$ and 0.0837, 0.0842 gm. $\text{H}_2\text{O}$ .
	$\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ . Calculated. C 43.74, H 5.51.
	Found. " 44.25, 44.21, " 5.09, 5.14.

The analytical results agree well with the empirical formula  $\text{Na}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ . The structure which we have proposed for the molecule sodium copper diethylbarbitate is to be found in the introduction.

## 2. Potassium Copper Diethylbarbitate, $\text{K}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ .

Potassium copper diethylbarbitate was obtained by a procedure analogous to that just described for the preparation of sodium copper diethylbarbitate. The potassium salt is deep rose in color, crystalline, and rather insoluble in water. It is hydrolyzed readily by water, and is decomposed by acids. The salt was analyzed for copper, sodium, and nitrogen.

0.3905, 0.5777 gm. substance:	0.0276, 0.0413 gm. Cu.
	$\text{K}_2\text{CuC}_{32}\text{H}_{48}\text{O}_{14}\text{N}_8$ . Calculated. Cu 6.98.
	Found. " 7.06, 7.14.

0.3905, 0.5777 gm. substance: 0.0778, 0.1122 gm.  $K_2SO_4$ .

$K_2CuC_{22}H_{42}O_{14}N_8$ . Calculated. K 8.60.

Found. " 8.94, 8.715.

0.3703, 0.3600 gm. substance: 17.45, 16.87 cc. 0.2 N  $H_2SO_4$ , factor 0.1932.

$K_2CuC_{22}H_{42}O_{14}N_8$ . Calculated. N 12.30.

Found. " 12.75, 12.67.

The empirical formula for potassium copper diethylbarbitate is considered to be  $K_2CuC_{22}H_{42}O_{14}N_8$  and its structure is undoubtedly analogous to that of sodium copper diethylbarbitate, already discussed.

#### SUMMARY.

1. Plans for an extensive investigation of the chemistry of the biuret reaction are outlined; the results of this study will be applied to problems of protein structure.

2. The present paper extends the study of the biuret reaction of simple acid imides to imides of the barbituric acid type.

3. Diethylbarbituric acid was found to be a typical acid imide in its behavior with the biuret reagents, forming a product of formula  $Me_2Cu(imide)_4 \cdot xH_2O$ .

4. The salts, sodium copper diethylbarbitate and potassium copper diethylbarbitate, were isolated and analyzed, empirical formulas were deduced from the analytical data, and a structure for the salts is proposed.

5. The rôle of the two biuret reagents, alkali and cupric ion, is discussed.

6. The atoms which are concerned in the biuret reaction of acid imides are designated.

# BLOOD SUGAR AND RESPIRATORY METABOLISM TIME CURVES OF NORMAL INDIVIDUALS, FOLLOWING SIMULTANEOUSLY ADMINISTERED GLUCOSE AND INSULIN.

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According to the literature, insulin is now being made use of in conditions other than diabetes. These include gastrointestinal disturbances in children, postoperative vomiting, vomiting during pregnancy, etc.; that is, conditions which lead to ketonuria and eventually, in the absence of treatment, to acidosis. In all instances recorded, the basis of its use appears to be the assumption that, as in diabetes, insulin administration results in utilization of carbohydrates at a rate greater than would be the case without it. Ketonuria is thus inhibited.

Theoretically, at least to the writer, the value of insulin in these conditions appears doubtful. There are no clinical or experimental data available demonstrating that insulin production is defective in these conditions, providing the individuals are not diabetics. Ketosis following vomiting in these conditions, though due to incomplete oxidation of fats, as in diabetes, differs, according to our present knowledge, from diabetes, in that it is not due to defective carbohydrate metabolism, but to lack of carbohydrates to be metabolized. The ketosis is a fasting phenomenon.

Further evidence opposing the view that there is defective insulin production in these non-diabetics is the fact that all of a number of blood sugar time curves obtained by the writer in such cases corresponded to the normal type. In the fasting state the blood sugars were normal. Following ingestion of glucose, the maximum peaks of the curves corresponded to blood sugars ranging between 0.16 and 0.18 per cent and, within 2½ to 3 hours,

the blood sugars returned to the normal levels. In addition to this, in some instances, there was other evidence of normal insulin production; namely, the curves "dipped" below the fasting level. These findings are diametrically opposite to those to be expected if insulin production were defective.

Experimental data concerning the influence of insulin upon normal animals are of interest here. It has been shown (1) that there is a striking difference between the reactions of normal and diabetic animals, from the point of view of glycogen formation. From the available experimental evidence Macleod concludes that "the difference is due to the fact that normal animals can, at all times, release from the pancreas a sufficient supply of insulin to metabolize, or polymerize, whatever amounts of carbohydrate may be present in the body. In other words, the endogenous supply of insulin is always at an optimum in the normal animal."

There is evidence, also based upon animal experimentation, that, in the normal animal, the response to simultaneously administered carbohydrates and insulin, as measured by the respiratory exchange, differs from that observed in diabetes. It appears that hypoglycemia tends to increase heat production and carbon dioxide excretion, regardless of carbohydrate oxidation. When this was taken into consideration, the alterations of the respiratory exchanges which were noted were insufficient to account for more than a small fraction of the sugar which disappeared. This phase of the subject is clearly discussed by Macleod in his chapter (No. 16) on "The Influence of Insulin on the Respiratory Exchange" (1).

In view of the above observations, it was decided to make a more detailed study of the carbohydrate metabolism in these conditions. Since such patients are non-diabetic, it was considered advisable, for comparative purposes, to study, firstly, the effects of insulin upon carbohydrate metabolism in *normal* individuals. This report is concerned with the results of these observations. The findings are of interest since they demonstrate that exogenous, that is administered, insulin not only does not appear to cause an increased rate of utilization of carbohydrates in normal subjects, but, temporarily at least, a decreased rate and altered mechanism are suggested.

*Method of Investigation.*

All subjects made use of were normal individuals following their usual dietary habits until the day of the test. To make more certain that we were dealing with normal individuals, blood sugar time curves were obtained in every case, following glucose ingestion. As a matter of fact, because of this procedure, two of the individuals selected for the work, though apparently normal otherwise, were rejected.

The method employed for studying carbohydrate metabolism in these cases was that which has been in use in this laboratory for a number of years. It was first applied in our laboratory to a study of renal glycosuria (2), and since then has become a standard procedure, hundreds of such tests having now been made. It consists of a simultaneous determination of blood sugar and respiratory metabolism time curves following glucose ingestion. That it is an ideal method of studying carbohydrate metabolism may be seen from the following observations.

Blood sugar time curves, when obtained without simultaneously determined respiratory metabolism curves, yield limited, though useful, information. The only indications of hyperglycemic response to glucose are (a) that the glucose ingested, or at least part of it, has been absorbed, and (b) the degree of hyperglycemia is some indication of tolerance, namely, an index of the disproportion between the rate at which glucose is being absorbed from the alimentary canal and the rate at which it is being utilized. Utilization may, however, be the result of storage or oxidation. The respiratory metabolism is an index of the degree of oxidation.

There are many necessary precautions to be taken in order to be reasonably certain that the respiratory quotients obtained during these tests approximate true metabolic conditions. As is well known, the respiratory quotient at the mouth is not necessarily the respiratory quotient in the body tissues. There are many factors, technical, psychological, *etc.*, which influence quotients and make difficult their interpretation. The possible sources of error and details of the precautions necessary were previously discussed by the writer (3). It is therefore unnecessary to repeat them here.

In addition to these precautions, other factors must be considered in the interpretation of respiratory quotient data when

*Data of Respiratory Metabolism and Blood Sugar Time Curves Following Simultaneously Administered Glucose and Insulin in Normal Individuals.*

Female subjects were used.

Subject.	Period. (1)	O <sub>2</sub> per hr. (2)	Total r.q. (3)	Calcu- lated N per hr.* (4)	Non- protein r.q.† (5)	Total calories per hr. (6)	Carbo- hydrate oxidized per hr. (7)	Percentage increase above basal.			Blood sugar.  per cent
								O <sub>2</sub> con- sump- tion.	CO <sub>2</sub> pro- duc- tion.	Heat pro- duc- tion.	
R. G., age 33 yrs. (20938-24). Ht. 152.5 cm., wt. 47.6 kilos, body surface 1.42 sq. m. B. M. R. +6.	Basal.	liters 11.79	0.713	gm. 0.313	0.695	55.29	0	-	-	-	0.102
	30	12.42	0.789	0.336	0.787	59.43	4.25	5	16	7	0.128
	60	11.91	0.748	0.317	0.721	56.44	0.67	1	6	2	0.172
	120	12.58	0.799	0.338	0.796	60.40	4.83	7	20	9	0.148
	180	12.39	0.763	0.333	0.671	58.88	0	5	15	6	0.092
B. M., age 50 yrs. (14831-25). Ht. 153.5 cm., wt. 59.0 kilos, body surface 1.56 sq. m. B. M. R. +1.	Basal.	11.52	0.804	0.313	0.803	55.31	4.42	-	-	-	0.116
	30	11.78	0.766	0.318	0.764	56.12	2.57	2	0	1	0.122
	60	11.64	0.810	0.317	0.811	56.02	4.96	1	2	1	0.099
	120	12.96	0.847	0.356	0.855	63.02	7.72	12	18	13	0.135
	180	13.42	0.797	0.364	0.795	64.43	4.59	16	15	16	0.084
C. M., age 40 yrs. Ht. 157.5 cm., wt. 42.8 kilos, body surface 1.39 sq. m. B. M. R. +11.	Basal.	11.58	0.789	0.314	0.785	55.46	3.48	-	-	-	0.111
	30	14.02	0.930	0.393	0.955	69.54	14.50	21	46	25	0.126
	60	13.64	0.909	0.381	0.931	67.33	12.89	17	36	21	0.105
	120	12.98	0.848	0.357	0.859	63.12	8.29	12	20	14	0.082
	180	12.50	0.791	0.339	0.788	59.86	4.28	8	8	8	0.054
O. T. M., age 38 yrs. (5572-23). Ht. 156.0 cm., wt. 48.4 kilos, body surface 1.45 sq. m. B. M. R. +1.	Basal.	11.09	0.827	0.304	0.831	53.65	5.66	-	-	-	0.096
	30	12.32	0.840	0.338	0.839	59.75	6.81	11	13	11	0.181
	60	12.16	0.789	0.329	0.786	58.13	4.17	9	5	8	0.169
	120	11.88	0.810	0.324	0.811	57.18	5.06	7	5	6	0.109
	180	11.62	0.859	0.321	0.869	56.65	7.92	5	9	5	0.060



M. M., age 29 yrs. (21154-24). Ht. 158.5 cm., wt. 54 kilos, body surface 1.54 sq. m. B. M. R. +13.	Basal. 30 60 120 180	13.40 13.60 13.42 14.64	0.808 0.805 0.899 0.869	0.365 0.331 0.374 0.404	0.809 0.807 0.918 0.882	64.49 65.29 66.08 71.54	5.71 5.79 12.08 10.62	1 1 0 9	1 11 18 11	1 2 11 8	0.112 0.188 0.151 0.183
G. M., age 30 yrs. (7293-25). Ht. 147.0 cm., wt. 44.0 kilos, body surface 1.34 sq. m. B. M. R. +5.	Basal. 30 60 120 180	10.65 12.32 12.46 11.75	0.839 0.768 0.789 0.809	0.292 0.332 0.338 0.320	0.840 0.840 0.762 0.810	69.62 51.65 58.69 59.67	7.96 5.89 2.69 4.27	7 15 17 10	7 6 10 6	8 14 15 9	0.128 0.111 0.129 0.100
M. D., age 27 yrs. Ht. 169.0 cm., wt. 57.0 kilos, body surface 1.65 sq. m. B. M. R. -2.	Basal. 30 60 120 180	12.59 13.06 15.55 13.62	0.745 0.789 0.829 0.775	0.338 0.354 0.425 0.367	0.775 0.733 0.787 0.768	53.63 59.66 62.54 64.88	2.91 1.22 4.47 3.54	6 4 24 8	6 10 37 12	4 5 26 9	0.063 0.113 0.125 0.143
C. A. R., age 45 yrs. (6493-28). Ht. 153.5 cm., wt. 53.4 kilos, body surface 1.55 sq. m. B. M. R. +2.	Basal. 30 60 120 180	12.15 13.62 13.42 14.14	0.804 0.765 0.769 0.789	0.330 0.366 0.362 0.382	0.805 0.757 0.753 0.787	58.32 64.72 63.93 67.53	5.15 2.98 2.38 4.84	12 10 16 13	7 6 10 17	11 10 16 16	0.094 0.181 0.135 0.139
	180	13.86	0.829	0.372	0.833	67.05	7.07	13	17	16	0.080

\*  $0.15 \times$  total calories

26.51

$$\frac{\uparrow \text{Total liters CO}_2 - (\text{gm. N} \times 4.76) \text{ liters CO}_2}{\text{Total liters O}_2 - (\text{gm. N} \times 5.91) \text{ liters O}_2} = \text{non-protein R. Q.}$$

insulin is given. As mentioned above, a fall in the blood sugar to the level at which hypoglycemic symptoms develop may result in respiratory quotient values greater than unity and the rise cannot wholly be accounted for by carbohydrate oxidation. Conversion of glucose into non-carbohydrate substances, production of acids, and alterations of the pH of the blood, *etc.*, are some of the possible causes. These have previously been discussed in great detail elsewhere (1).

Confidence in the procedure we made use of is enhanced by the fact that our results of studies of renal glycosuria (2), and other studies (4), were similar to those made at a later date with the respiratory calorimeter at the Russell Sage Institute of Pathology. The latter include the observation of Ladd and Richardson on renal glycosuria (5), and McClellan, Biasotti, and Hannon on the metabolism of dihydroxyacetone (6).

The standards employed for comparison are those generally accepted. They are as follows:

The average respiratory quotient during the fasting period is 0.82 (0.83 for males and 0.81 for females) (7). After the administration of 100 gm. of glucose (8), an increase rarely fails to occur within 30 minutes. The average maximum increase is about 0.12. In some instances a slight decrease may be noted, such as conditions which deplete glycogen (exercise, fasting, *etc.*). Benedict and Carpenter found a fairly consistent picture in the ten cases observed. The lowest, highest, and average peaks of carbon dioxide production were respectively 12, 35, and 25 per cent above the basal period; of oxygen consumption 3, 22, and 12 per cent; of heat production 6, 24, and 14 per cent, and of the respiratory quotients 0.07, 0.17, and 0.12 per cent. The average time of appearance of the maximum effects was within 2 hours.

In this study a Tissot gasometer, Haldane gas analysis apparatus, and half face Siebe-Gorman mask were used. In each case the respiratory metabolism and blood sugar were determined in the basal metabolic state and then again, 30, 60, 120, and 180 minutes after the ingestion of 100 gm. of glucose and the administration of 25 units of insulin. The insulin was administered subcutaneously and simultaneously with the ingestion of glucose. Samples of blood were taken at the end of each period for blood sugar analyses. *Since the individuals were normal, and because*

of the generally recognized uncertainty that the urine nitrogen obtained at short intervals of time may not represent the protein metabolism at the time of the test, no nitrogen determinations were made. 15 per cent of the total metabolism was attributed to protein. For the validity of this procedure, there appear to be sufficient data (9). The remaining calories, assumed to be derived from carbohydrates and fats, were apportioned according to the Zuntz-Schumburg tables, as modified by Williams, Riche, and Lusk (10). The data are shown in detail in Table I and briefly summarized in Table II.

TABLE II.  
*Percentage Increases of Basal Periods.*

Subjects.	R.Q.*			O <sub>2</sub> consumption.			Heat production.		
	Low.	High.	Average.	Low.	High.	Average.	Low.	High.	Average.
Normal.	0.07	0.17	0.12	3	22	12	6	24	14
Insulin.	0.01	0.14	0.06	1	24	9	1	26	10

\* R.Q. values are absolute increases and not expressed as percentages.

#### DISCUSSION.

Taken as a whole, the blood sugar time curves obtained are of interest in that they do not correspond to the typical responses of normal individuals to glucose ingestion. As is well known, the blood sugar increases rapidly, reaches a peak at the end of the 30 minute period, and decreases rapidly thereafter. All these individuals were normal. In spite of this, a number of irregularities were observed in the time curves after the simultaneous administration of glucose and insulin. The curves of the subjects R. G. and M. D. fail to show the abrupt increases; those of subjects R. G., O. T. M., M. M., and C. A. R. show gradual declines, and irregularity is shown in subjects B. M. and M. M. in that there is a reappearance of hyperglycemia at the 120 minute period.

Though one would hardly regard the above findings as distinctly abnormal, the fact remains that such irregularities as noted, though they occur, do so rather uncommonly. In our laboratory, we have

now had experience with many thousands of blood sugar determinations and with over 1000 such curves obtained with the same dose of sugar, the same type of sugar, and the same laboratory technique and, though such curves as we note here have been met with occasionally, it is remarkable that of such a small group of subjects, selected at random, so many suggestive abnormalities should be found. The possibility is suggested that the insulin administered in some way was responsible for this, either by altering the rate of oxidation or the rate of storage. It is believed that experimental errors are eliminated as a possible cause.

In Table II is given a synopsis of the detailed data recorded in Table I. When these findings are compared with the normal individual a striking difference is noted. Thus, the values of the low, high, and average respiratory quotients are definitely below the normal. The low and average values of  $\text{CO}_2$  production are also definitely below the normal, though the highest value is greater. The same applies to the values corresponding to the oxygen consumption and heat production.

There is a definite decrease of the rate of oxidation of carbohydrates. This is shown in Column 7 of Table I. The average amount of carbohydrates oxidized during the basal periods was 3.9 gm. per hour, whereas the average amount after the administration of glucose and insulin was 6.4 gm. In two cases only were normal values approached; namely, in subject C. M., who oxidized an average of 10 gm. of carbohydrates per hour, and subject M. M., who oxidized 9.1 gm.

Interpretation of the above data appears to offer little difficulty. With the exception of the blood sugar values found at the 180 minute period in the cases of subjects C. M., O. T. M., and G. M., none of the other values suggest hypoglycemia. Since there was no hypoglycemia, it appears, in view of the above observations, reasonable to assume that any increases of respiratory quotients and total metabolism were the result of carbohydrate oxidation. Further evidence of this is the fact that when each non-protein respiratory quotient is correlated with the corresponding heat production, an increase of the former corresponds with an increase of the latter.

## CONCLUSIONS.

In view of the above findings and the data available on animal experimentation, it is suggested that, unlike its effect in diabetes, insulin does not increase the rate of oxidation of administered carbohydrates in normal individuals. As a matter of fact, a decreased rate was observed. This suggests that insulin not only does not enhance oxidation of sugar in the normal individual, but in some as yet unexplained way interferes with the normal mechanism. Similar studies are now being made in a series of non-diabetic individuals with conditions resulting in ketonuria.

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## THE NATURE OF BLOOD SUGAR.

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In 1909 Hollinger reported (1) that when performing parallel determinations of blood sugar by two different analytical methods he obtained different sugar values. In one of the methods an alkaline mercury solution (Knapp), in the other a copper reagent was used, of which the mercury solution yielded always lower results. This was interpreted by Hollinger as being due to the presence in blood of some sugar other than glucose. 2 years ago Folin and Svedberg (2) revived this conception on the ground of similar findings. These authors determined the fermentable reducing substances in blood by the Folin-Wu method, and at the same time with Folin's modified reagent, and as a result of the discrepancies found between the two sets of values, they were led to the same conclusion as Hollinger.

At that time we had made similar observations in parallel determinations of blood sugar in Folin-Wu filtrates, by the Shaffer-Hartmann method and by an alkaline potassium ferricyanide reagent. The ferricyanide reagent yielded consistently higher values as shown in the following examples:

<i>Blood Sugar, Mg. Per Cent.</i>	
<i>Copper reagent.</i>	<i>Ferricyanide reagent.</i>
107	127
92	110
216	236
137	163
140	173
260	282
256	289

From a few parallel determinations of the residual reduction by both methods we were much inclined to ascribe these discrepan-

cies entirely to the non-sugar fraction of the total reduction values, yet our results were too inconsistent and confusing to permit of such conclusion. Eventually, after a sufficiently reliable technique had been developed in this laboratory for the determination of the reducing non-sugars, we undertook a more detailed investigation of the question. The procedure of Folin and Svedberg, however, was not duplicated. In the main series of our comparative sugar determinations we employed the Shaffer-Hartmann method and the Folin-Wu method, the latter with some modifications to be described presently. In another group of experiments parallel determinations were performed by the Shaffer-Hartmann method and a ferricyanide method, based upon the Hagedorn-Jensen procedure. Finally, in nine cases all three of these methods were applied.

#### *Colorimetric Method.*

We used the Folin method with two alterations.

1. The more essential of the two is a modification of the copper reagent; it is a solution containing 20 gm. of  $\text{Na}_2\text{CO}_3$  (anhydrous), 25 gm. of  $\text{NaHCO}_3$ , 25 gm. of Rochelle salt, 7 gm. of  $\text{CuSO}_4$  (crystalline) per liter. This variation of Folin's copper reagent may as well be regarded as an adaptation of the modified Shaffer-Hartmann reagent (3) for colorimetric work by omission of the ingredients serving the iodometric determination of cuprous oxide. It was preferred to both the Folin-Wu and the Folin reagent for two reasons. First, as has been demonstrated by Somogyi (3), copper reagents containing carbonate and bicarbonate in the ratio as employed in this reagent yield maximum reduction values, a property highly desirable and useful in the determination of the very small amounts of reducing non-sugars in blood and blood plasma. Second, this carbonate:bicarbonate ratio represents about the optimum range of buffer action, whereby the reagent is rendered less susceptible to the influence of factors that may affect the pH of the reaction mixture, and thus obviates the necessity of neutralizing Folin-Wu filtrates for the determination of sugar. The figures in Table I show the sensitiveness of our reagent as compared with the Folin-Wu and Folin reagents. As can be seen, our reagent gives about 50 per cent more reduction than Folin's, and about twice as



much as the Folin-Wu reagent, at the low sugar concentration corresponding to the amount of reducing non-sugars in blood. At ordinary concentration of blood sugar, where increased sensitiveness is of no practical significance, the differences are small.

2. In place of the Folin-Wu sugar tubes we employ ordinary test-tubes, covered with glass bulbs or small funnels during heating, as in the Shaffer-Hartmann method. The reoxidation of cuprous oxide is slightly greater in covered test-tubes than in Folin-Wu tubes, but this difference—less than 1 mm. in colorimetric readings—does not affect the accuracy of the results. The accurate and well reproducible results furnished by the Shaffer-Hartmann method are ample proof of the fact that the reoxidation in this procedure represents a quantity of rather consistent character; moreover, in colorimetric work standard and unknown are exposed to the same standardized conditions, whereby the influence of reoxidation is ruled out, and parallel determinations yield equally good checks whether carried out in Folin-Wu tubes or covered test-tubes.

The following examples illustrate the magnitude of differences in the extent of reoxidation occurring in the two kinds of tubes.

Concentration of glucose solution.	Colorimetric readings.	
	Folin-Wu tubes.	Covered test-tubes.
<i>per cent</i>	<i>mm.</i>	<i>mm.</i>
0.002	25.0	25.2
0.010	20.0	20.5
0.010	20.0	{ 20.7
		{ 20.7
0.020	15.0	15.7
0.020	15.0	{ 15.7
		{ 15.8

Thus, without incurring by this change any disadvantage whatsoever, the method was made more elastic and adaptable to various needs in our work. For the determination of apparent sugar 2 cc. of blood filtrate are heated with 2 cc. of the copper reagent in 16 × 150 mm. test-tubes which bear a filed mark at 10 and 25 cc. If the concentration of the blood sugar is 300 mg. per cent or higher, the color is so deep that dilution to 50 cc. is necessary, unless one prefers to dilute the blood filtrate prior to the sugar

determination. At very low sugar concentrations, for instance 10 mg. per cent (1 mg. per cent in 1:10 protein-free filtrates), the reaction mixture is diluted only to 10 cc. as suggested by Benedict (4), or the following technique may be used conveniently. In a 25 × 200 mm. test-tube 5 cc. of filtrate are heated with 5 cc. of copper reagent, and after cooling 5 cc. of the Folin-Wu color reagent are added. Preceding the transfer of the liquid into the colorimeter cup the bulk of the CO<sub>2</sub> is removed by vigorous agitation. At still lower concentrations 10 cc. of filtrate are heated with 5 cc. of copper reagent, and after cooling 5 cc. of color reagent are added. These proportions were used for fermented 1:10 filtrates of blood plasma in which the amount

TABLE I.

Concentration of glucose solution.	Colorimeter readings.		
	Folin-Wu reagent.	Folin reagent.	New reagent.
<i>per cent</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
0.025	20.0	19.5	17.6
0.020		15.0	13.0
0.020	15.0	13.8	12.4
0.010	20.0	15.7	15.0
0.001	30.0	20.0	13.3
0.001		25.0	17.0
0.001		25.0	15.5
0.001	30.0		18.4

of reducing substances is equivalent to 0.3 to 0.5 mg. per cent glucose. No special adjustment of the volume is necessary at these low concentrations, as the changes in volume during heating are too small to affect the results. One rule to be strictly observed is that in standard and unknown the volume of the sugar solution and the quantity of reagents added must be identical. Aside from this, a variety of individual alterations may be applied to technical details of the Folin-Wu method.

#### *Ferricyanide Method.*

The Hagedorn-Jensen potassium ferricyanide reagent is devised for micro determinations in 12 cc. of protein-free filtrate obtained from 0.1 cc. of blood. In this form it is unfit for our work involv-

ing determinations of apparent sugar and reducing non-sugar values in 2 and 5 cc. portions of the same Folin-Wu filtrates that were used for sugar determinations also by the Shaffer-Hartmann and the colorimetric methods. In the present experiments we made use of a modified ferricyanide reagent, prepared on August 31, 1926. It contains 20 gm. of  $\text{Na}_2\text{CO}_3$  (anhydrous), 25 gm. of  $\text{NaHCO}_3$ , 5.8 gm. of  $\text{K}_3\text{Fe}(\text{CN})_6$  per liter. Either 2 cc. of this reagent with 2 cc. of blood filtrate in  $18 \times 150$  mm. test-tubes, or 5 cc. of the reagent with 5 cc. of blood filtrates in  $25 \times 200$  mm. test-tubes, are heated in boiling water for 15 minutes. After cooling 1 cc. of a 5 per cent KI solution, then a volume equal to that of the ferricyanide (2 or 5 cc.) of a solution containing 15 per cent of  $\text{ZnSO}_4$  and 20 per cent of NaCl are added; finally the mixture is acidified by the addition of 2 or 5 cc. respectively of 0.75 N  $\text{H}_2\text{SO}_4$ , and titrated against 0.005 N thiosulfate. A blank sample of the reagent is carried through the same procedure, to determine its iodine value. The reduction equivalent of the reagent is determined with pure glucose solutions of known concentrations. For the reagent employed in the present experiments, 1 cc. of 0.005 N iodine corresponds to 0.1397 mg. of glucose.

This technique, as also the deviations from Folin's procedure described above, were devised to meet the special requirements arising from the nature of our present experiments, and are not offered as additions to the numerous useful methods available for the determination of blood sugar in general.

#### EXPERIMENTAL.

Blood, corpuscles, or plasma were deproteinized according to Folin and Wu in quantities to furnish sufficient filtrate for all determinations to be carried out in an experiment. For corpuscles and plasma the precipitants were used in proportions previously described by Somogyi (5). About one-half of each filtrate was freed from sugar by washed yeast (5) for the determination of reducing non-sugars.

In thirty-seven samples of whole blood, four of corpuscles, and four of plasma, apparent sugar and reducing non-sugars were determined by the Shaffer-Hartmann and by the colorimetric methods. The results, given in Tables II and III, show that the colorimetric method yields, on an average, nearly 12 mg. per cent

lower values than the Shaffer-Hartmann method. The same difference, however, is found in the reducing non-sugars, determined by the two methods, so that the two sets of true sugar

TABLE II.  
*True Sugar Content of Non-Diabetic Human Blood, Estimated by Two Different Analytical Methods.*

Specimen No.		Shaffer-Hartmann method.			Colorimetric method.		
		Apparent sugar.	Reducing non-sugar.	True sugar.	Apparent sugar.	Reducing non-sugar.	True sugar.
		Mg. per cent in terms of glucose.					
1	Non-diabetic blood.	103	24	79	88	10	78
2		101	24	77	88	10	78
3		112	25	87	102	10	92
4		107	30	77	89	13	76
5		124	27	97	110	13	97
6		112	22	90	108	10	98
7		86	20	66	76	10	66
8		111	22	89	98	11	87
9		76	18	58	71	9	62
10		102	18	84	94	10	84
11		109	20	89	97	10	87
12		107	21	86	90	12	78
13		112	22	90	102	10	92
14		94	20	74	82	11	71
15		113	22	91	101	12	89
Average.....		104.6	22.3	82.3	93	10.7	82.3
16	Corpuscles.	107	37	70	83	15	68
17	“	106	37	69	87	15	72
18	“	118	42	76	101	18	83
19	“	113	38	75	99	23	76
20	Plasma.	99	10	89	93	4	89
21	“	96	10	86	89	4	85
22	“	106	9	97	103	4	99
23	“	70	6	64	69	4	65
Average.....				78.2			79.6

values are substantially the same. The almost perfect identity of the averages strengthens our view that the discrepancies between the true sugar values of individual samples are due exclusively to

technical causes. These individual discrepancies are somewhat more pronounced in the diabetic specimens of Table III, but even here we find only one single case in which the difference is above 10 mg. per cent (Specimen 31). Probably the lower degree of

TABLE III.

*True Sugar Content of Diabetic Human Blood, Estimated by Two Different Analytical Methods.*

Specimen No.	Shaffer-Hartmann method.			Colorimetric method.		
	Apparent sugar.	Reducing non-sugar.	True sugar.	Apparent sugar.	Reducing non-sugar.	True sugar.
Mg. per cent in terms of glucose.						
24	174	21	153	161	13	148
25	123	24	99	112	12	100
26	139	27	112	129	13	116
27	279	22	257	256	10	246
28	180	17	163	177	10	167
29	163	24	139	145	16	129
30	175	22	153	152	9	143
31	203	21	182	180	12	168
32	373	18	355	361	9	352
33	158	17	141	158	10	148
34	222	19	203	221	11	210
35	155	18	137	146	10	136
36	204	20	184	197	12	185
37	186	17	169	187	11	176
38	269	19	250	266	11	255
39	165	20	145	159	10	149
40	162	21	141	149	10	139
41	123	22	101	109	10	99
42	155	22	133	143	11	132
43	126	22	104	119	11	108
44	187	23	164	174	13	161
45	154	23	131	140	11	129
Average.....	164.4			163.5		

accuracy of the colorimetric method at higher sugar concentrations is responsible for the greater discrepancies. We found differences of 10 to 20 mg. per cent, and even greater in matching against one another, by the colorimetric method, two or three portions of one and the same 25 to 30 mg. per cent pure glucose solution. This

source of inaccuracy proved to be beyond our control, but in every other respect we applied scrupulous care in technical details in order to preclude experimental errors as far as possible. Thus, the standard sugar solutions were frequently checked by the Shaffer-

TABLE IV.

*True Sugar Content of Non-Diabetic and of Diabetic Blood, Estimated by Three Different Analytical Methods.*

Specimen No.		Shaffer-Hartmann method.			Ferricyanide method.			Colorimetric method.		
		Apparent sugar.	Reducing non-sugar.	True sugar.	Apparent sugar.	Reducing non-sugar.	True sugar.	Apparent sugar.	Reducing non-sugar.	True sugar.
		Mg. per cent in terms of glucose.								
12	Normal human blood.	107	21	86	127	43	84	90	12	78
13		112	22	90	126	36	90	102	10	92
14		94	20	74	112	39	73	82	11	71
15		113	22	91	125	38	87	101	12	89
41	Diabetic human blood.	123	22	101	142	37	105	109	10	99
42		155	22	133	175	40	135	143	11	132
43		126	22	104	143	40	103	119	11	108
44		187	23	164	203	42	161	174	13	161
45		154	23	131	170	43	127	140	11	129
46		226	17	209	246	32	214			
47		249	17	232	271	35	236			
48		117	19	98	141	38	103			
49		192	23	169	207	37	170			
50		146	24	122	161	44	117			
51	Calf blood.	112	21	91	130	40	90			
52	“ plasma.	119	8	111	126	15	111			
53	“ corpuscles.	101	37	64	130	70	60			
Average.....		143	21	122	161	39.5	121.5			
“ *.....		180	22	108	147	40	107	118	11	107

\* Average for the first nine specimens above.

Hartmann method; the sugar content of the blood filtrates was first determined titrimetrically so that in subsequent colorimetric work standards could be chosen to approximate closely the sugar content of the blood filtrates.

The agreement between true sugar values is very good in parallel determinations by the Shaffer-Hartmann and the ferricyanide methods, a circumstance which supports our opinion that the greater discrepancies occasionally found by the colorimetric method are entirely due to the diminished reliability of this method at higher levels of blood sugar. The Shaffer-Hartmann and the ferricyanide methods yield true sugar values checking within 0 to 5 mg. per cent in diabetic hyperglycemia as well as at normal sugar levels, and the difference between the averages of seventeen determinations is only 0.5 mg. per cent. There is a very pronounced discrepancy between the two sets of apparent sugar values; but as can be seen, this is fully accounted for by the difference between the reducing non-sugars as obtained by the two methods.

The first nine samples recorded in Table IV were analyzed by three methods. The three sets of apparent sugar values so obtained exhibit marked discrepancies, the ferricyanide method yielding on an average 39 mg. per cent higher results than the colorimetric, and 17 mg. per cent higher than the Shaffer-Hartmann method. These differences, however, are identical with those found in the reducing non-sugars, and the average values for true sugar are identical. Even the differences in the individual samples are without exception small enough to be considered as of technical origin.

Thus our experimental evidence, derived from the analysis of 53 specimens, corroborates Benedict's recent observations (4) obtained by a procedure essentially different from ours. Benedict determined the fermentable sugar in blood by the Folin-Wu method and by his own new method which furnishes true sugar values directly, and was "unable to find any indication of the presence of non-glucose fermentable sugar" in human blood.

The findings of Folin and Svedberg, contrary to this, may be due partly to causes adduced by Benedict, partly to the inconsistency of the colorimetric technique at relatively high concentrations of glucose, and to some extent to the use of unwashed yeast and of kaolin in the fermentation process.

## SUMMARY.

Determinations of blood sugar by different analytical methods produce markedly different results, if the apparent sugar (total reduction) values are accepted as representing the actual sugar content. From this Hollinger inferred that blood contains some sugar other than glucose.

Folin and Svedberg derived the same conclusion from determinations of fermentable true sugar in blood by two methods. Determinations of true sugar by three different methods, reported in this paper, yielded essentially identical values; these results do not warrant the assumption that blood contains measurable amounts of any fermentable sugar other than glucose.

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## A CONTRIBUTION TO THE CHEMISTRY OF GRAPE PIGMENTS.

### V. THE ANTHOCYANS IN IVES GRAPES.

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#### INTRODUCTION.

The pigments occurring in a number of varieties of grapes have been examined in this laboratory during the past few years. The grapes included in these investigations were three varieties of pure American stock representing *Vitis labrusca*, *aestivalis*, and *riparia*, and two hybrids containing the *Vitis rupestris*-*Vitis vinifera* and *Vitis labrusca*-*Vitis vinifera* strains.

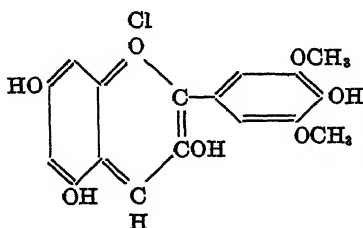
The pigments present in the American varieties, Concord, Norton, and Clinton grapes (1) were found to be glucosides that were very similar to and probably identical with the glucoside, ampelopsin, found by Willstätter and Zollinger (2) in the wild *Ampelopsis quinquefolia*.

The pigments occurring in the hybrids that were examined, namely Seibel and Isabella grapes (3), were found to be identical with the glucoside, oenin, first isolated from the dark blue European grape *Vitis vinifera* by Willstätter and Zollinger (4).

The chief difference between the anthocyanins occurring in the pure American varieties and in the hybrids mentioned above, lies in the higher percentage of methoxyl groups in the pigments isolated from the grapes containing the strain of *Vitis vinifera*. The investigations showed that these pigments were glucosides of mono- and dimethoxydelphinidin and that the anthocyanin oenin, which occurs in *Vitis vinifera*, is inherited by the hybrids.

The dimethoxydelphinidin isolated from Isabella grapes, after acetylation and oxidation with neutral permanganate, yielded

syringic acid. It was evident therefore that oenidin chloride possessed the following structure:



instead of the isomeric formulas proposed by Willstätter and Zolinger (2). Karrer and Widmer (5) have recently shown that oenidin chloride, obtained from the European grape *Vitis vinifera*, also yields syringic acid on treatment with alkali.

The purpose of the present investigation was to prepare a larger quantity of the pigment from another American grape in order to make a more complete examination of this type of anthocyan pigment. The dark blue Ives grape was selected because it evidently contained an abundant supply of coloring matter. According to Hedrick (6) the Ives grape is classified as a practically pure *Vitis labrusca* with possibly some admixture of *Vitis æstivalis*. The Ives grape should therefore contain a pigment very similar to that found in the Concord grape, since the latter is known to be pure *Vitis labrusca*.

The pigment from the Ives grape, however, differs markedly from the other anthocyanins that have been isolated in this laboratory. The original pigment was non-crystalline and contained some amorphous material from which it could only be freed by repeated precipitation by ether from a methyl alcoholic hydrochloric acid solution. The chloride of the glucoside could not be obtained in crystalline form and the anthocyanidin chloride, obtained by hydrolysis of the glucoside, was only partly crystalline. The large amount of pigment prepared, however, has made possible a thorough examination and the results show that the pigment isolated from the Ives grape is a mixture of the following components. (a) Anthocyanidin chloride. This non-glucoside fraction was present to the extent of about 3.7 per cent. (b) Anthocyanin chloride. This component consisted of monoglucosides of

mono- and dimethoxydelphinidin chloride. (c) *p*-Hydroxycinnamoyl derivatives of the above glucosides present to only a very low percentage.

The small amount of the *p*-hydroxycinnamoyl derivative probably accounts for the amorphous nature of the glucoside, since it has been found that the anthocyanin monardin (7) is non-crystalline and it also yields *p*-hydroxycinnamic acid on hydrolysis.

Attempts were made to separate the mono- and dimethoxydelphinidin glucosides by fractional crystallization of their picrates according to the procedure of Karrer and Widmer (7), but, as shown in the experimental part, no definite separation can be obtained by this method.

The identity of the glucosides obtained from the other American grapes with ampelopsin was indicated by the fact that they gave the beautiful blue coloration with ferric chloride described by Willstätter and Zollinger as characteristic of ampelopsin, but which is not given by oenin. The glucoside isolated from Ives grapes also gave this ferric chloride reaction, indicating that it is an American grape. The facts, however, that it gives syringic acid on oxidation and that it contains *p*-hydroxycinnamic acid would indicate that the Ives grape is not a pure *Vitis labrusca* but probably contains some *Vitis vinifera* mixed with still another strain.

#### EXPERIMENTAL.

*Isolation of Pigment as Picrate.*—The skins of 500 pounds of freshly picked ripe Ives grapes were separated from the pulp by hand. The skins were pressed first in an ordinary wine-press and then in a hydraulic press. The pressed skins which weighed 33 kilos were digested in about 30 liters of 1 per cent hydrochloric acid. After the mixture had stood overnight it was run through a fruit screw-press in order to macerate the skins and bring the pigment into solution. The magma was then pressed in a hydraulic press and the expressed juice filtered through a layer of paper pulp. The press cake was stirred up with 20 liters of 0.5 per cent hydrochloric acid and the above procedure repeated. The clear but intensely dark red filtrates, which measured about 60 liters, were combined, warmed to 40°, and 700 gm. of finely powdered picric acid were dissolved in the solution. On cooling there separated slowly a large amount of a dark red amorphous material

that collected as a gummy mass on the bottom of the container. After the mixture had stood for 2 days the supernatant liquid was decanted and the precipitate was freed as much as possible from the mother liquor by centrifuging and washing with a little cold water. The crude picrate after it had been dried *in vacuo* over sulfuric acid formed a nearly black, hard brittle mass that could easily be powdered. It weighed about 600 gm.

*Preparation of Anthocyanin Chloride.*—The crude dried picrate was converted to the chloride as follows: 100 gm. lots of the picrate were dissolved in 600 cc. of absolute methyl alcohol containing 5 per cent of dry hydrochloric acid. The insoluble material was filtered off and the intensely deep red solution was poured with vigorous mechanical stirring into 3 liters of dry ether. The anthocyanin chloride was precipitated as a deep red amorphous powder. The product was filtered off and washed thoroughly with ether in order to remove the picric acid. The substance was then dried in a vacuum desiccator over sulfuric acid. About 20 gm. of crude glucoside chloride were obtained from 100 gm. of picrate.

All attempts to obtain the glucoside in a crystalline form failed. For analysis it was precipitated twice by ether from solutions in methyl alcohol containing hydrochloric acid. The substance was dried at 105° in a vacuum over phosphorus pentoxide.

*Analysis.*

	C	H	Cl	CH <sub>3</sub> O
Found.....	51.40	4.78	5.39	5.90
Calculated for C <sub>23</sub> H <sub>23</sub> O <sub>12</sub> Cl (1 methoxyl).....	51.35	4.50	6.90	5.87
Calculated for C <sub>23</sub> H <sub>23</sub> O <sub>12</sub> Cl (2 methoxyls).....	52.22	4.73	6.72	11.73

The above analyses would indicate that the pigment is a monoglucoside of a monomethoxydelphinidin chloride but as will be seen from subsequent data, it is really a mixture. The substance was optically active but the rotation could not be determined with any great degree of accuracy because the solutions were too highly colored. A solution of 25 mg. in 100 cc. of 0.5 per cent hydrochloric acid was examined in a 1 dm. tube with white light. Observed rotation  $-0.07^\circ \pm 0.01^\circ$ .  $[\alpha]^{25} = -280^\circ \pm 40^\circ$ .

The distribution coefficient of the pigment between amyl alcohol and water was determined according to the procedure of

Willstätter and Zollinger (8), and found to be 51.8. This value does not agree with Willstätter and Zollinger's value for the monoglucoside oenin (10.4). The difference is due to the presence of non-glucoside pigment. The amount of free anthocyanidin present was determined by extracting the amyl alcohol with 0.5 per cent hydrochloric acid until the aqueous extract was no longer colored. The aqueous hydrochloric acid removes the anthocyanin while the anthocyanidin is retained in the amyl alcohol. It was found by comparison with standard amyl alcoholic solution of anthocyanidin, that 3.68 per cent of the original pigment was present as non-glucoside.

TABLE I.

*Composition of Anthocyanin Fractions Obtained by Crystallization of Crude Picrate.*

Fraction No.	Weight.	Methoxyl.	Chlorine.
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
1	2.5	6.74	5.92
2	1.5	6.36	5.43
3	5.0	6.49	6.15
4	7.0	5.71	5.16
5	0.8	8.53	6.72
6	1.9	4.54	4.80
Theory, $C_{23}H_{23}O_{12}Cl$ .....		5.87	6.90
" $C_{23}H_{23}O_{12}Cl$ .....		11.73	6.72

*Fractionation of Anthocyanin Picrate.*—Since the chloride of the glucoside could not be obtained in crystalline form an attempt was made to purify it by converting 30 gm. of the amorphous glucoside back into the picrate and separating the latter into six fractions by crystallization from water. The different fractions of the picrate were then reconverted into the chloride by the method already described. The composition of the various fractions is shown in Table I.

Fraction 1 was the most soluble and Fraction 5 was the least soluble, while Fraction 6 represents that portion of the original picrate which was insoluble in hot water.

The results of the fractionation were disappointing so far as the separation of pure compounds was concerned. Fraction 5 was

the only product that gave a definitely crystalline picrate. While its methoxyl content had increased, indicating that a concentration of dimethoxydelphinidin had taken place, it is evident that it still contained a large proportion of monomethoxydelphinidin. The small amount of material in this fraction precluded any further efforts to obtain a pure oenin from this fraction. The low values obtained in the halogen determinations were due to the fact that a small part of the anthocyanin was present as the *p*-hydroxycinnamoyl derivative, and this grouping has not been included in calculating the theoretical values which are included only for the sake of comparison.

*Hydrolysis of Anthocyanin Chloride.*—For hydrolysis, 5 gm. of the pigment from Fraction 4 above were dissolved in 100 cc. of warm 0.5 per cent hydrochloric acid, the solution was heated to boiling, and 100 cc. of concentrated hydrochloric acid added. The solution was boiled 3 minutes. The anthocyanidin chloride separated out as the solution cooled, and was filtered off, and washed with cold dilute hydrochloric acid. The yield was 2.6 gm. of material which was only partly crystalline and possessed no definite melting point. All attempts to recrystallize this substance gave only amorphous products. It was analyzed after drying at 105° over phosphorus pentoxide. The loss in weight on drying was 4.70 per cent. Calculated for the monohydrate  $C_{16}H_{13}O_7Cl \cdot H_2O$ ,  $H_2O$  4.86 per cent.

*Analysis.*

	C	H	Cl	CH <sub>3</sub> O
Found.....	54.84	4.11	8.63	9.87
Calculated for $C_{16}H_{13}O_7Cl$ (1 methoxyl).....	54.47	3.72	10.06	8.78
Calculated for $C_{17}H_{15}O_7Cl$ (2 methoxyls).....	55.66	4.09	9.68	16.92

The above methoxyl determination shows that this fraction still contained some dimethoxydelphinidin and that a complete separation had not been obtained by fractionation of the picrates.

*Isolation of p-Hydroxycinnamic Acid.*—The filtrate from the anthocyanidin chloride was extracted four times with 100 cc. portions of ether. After distilling off the ether a yellow residue was obtained which yielded white needles after two crystalliza-

tions from hot water and norit. M. p. 213–214°. For analysis it was converted to the silver salt.

*Analysis.* 0.2105 gm. substance; 0.0847 gm. Ag.

Calculated for  $C_6H_7O_3Ag$  (270.8). Ag 39.80 per cent. Found, Ag 40.23 per cent.

The substance was identified as *p*-hydroxycinnamic acid by comparison with a known sample of this acid prepared by carrying out Perkin's reaction on *p*-hydroxybenzaldehyde (9). A mixed melting point on the two samples showed no depression.

*Isolation and Identification of Glucose.*—The dark red filtrate from the anthocyanidin chloride, after extraction of the *p*-hydroxycinnamic acid, was diluted to 250 cc. with water. A 50 cc. portion was neutralized with sodium carbonate, 10 cc. of lead acetate solution were added, and a few drops of acetic acid until the solution was slightly acid to litmus. This procedure precipitates the pigment left in solution as the blue lead salt. After filtering off the lead precipitate the solution was diluted to 100 cc. and the excess lead removed by adding dry disodium phosphate (10). The solution was filtered and the filtrate was used for the quantitative determination of reducing sugars according to the method of Shaffer and Hartmann (11). Calculated for monoglucoside of monomethoxydelphinidin  $C_{22}H_{23}O_{12}Cl$  (514.5). Glucose 34.98 per cent, found 28.80 per cent. The low result is probably due to the destruction of part of the glucose by the hot hydrochloric acid. Another portion of the clarified solution was polarized and the specific rotation calculated with the copper reduction value found above. Found  $[\alpha]_D^{25} = + 53.8^\circ$ ,  $[\alpha]_D^{25}$  for glucose = + 52.8°.

*Isolation of Glucosazone.*—Another 100 cc. portion of the clarified filtrate was treated with 5 gm. of phenylhydrazine, 5 cc. of acetic acid, 10 gm. of sodium acetate, and heated in a boiling water bath for 30 minutes. The phenylglucosazone crystallized out in characteristic needles which melted at 204–205°.

*Decomposition of Anthocyanin by Alkali.*—Anthocyanin chloride, 5 gm., was dissolved in 60 cc. of 12 per cent sodium hydroxide and the solution was allowed to stand at room temperature for 3 hours. The solution was acidified and extracted with ether. On evaporation of the ether a residue was obtained which after it had been

crystallized twice from water melted at 213–214°. The substance was *p*-hydroxycinnamic acid as shown by the fact that when mixed with a pure sample of this acid there was no depression of the melting point.

The aqueous solution, after it had been extracted with ether, as mentioned above, was neutralized and solid sodium hydroxide was added to make a 12 per cent solution. The solution was then boiled in an atmosphere of nitrogen for 3 hours in an attempt to obtain syringic acid as described by Karrer and Widmer (5). After the reaction mixture had cooled it was acidified and extracted with ether. On evaporation of the ether only a very slight residue was obtained from which no definite substance could be isolated.

TABLE II.  
*Composition of Anthocyanidin Acetates.*

Product.	M. p.	Color.	CH <sub>3</sub> CO	CH <sub>3</sub> O
	°C.		per cent	per cent
α-Acetate.....	102–104	Tan.	34.43	7.35
β-Acetate.....	220–230	Purple.	34.67	6.91
Calculated for:				
Tetraacetyl dimethoxydelphinidin....			34.46	12.50
“ monomethoxydelphinidin.			35.55	6.41
Pentaacetyl “			39.84	5.73
Triacetyl dimethoxydelphinidin.....			28.30	13.60

*Acetylation of Anthocyanidin Chloride.*—The anthocyanidin will yield two acetyl derivatives, depending upon the method of preparation, that differ in appearance and in properties but not in composition. When the substance is acetylated according to the method of Einhorn and Hollandt (12) a tan or straw-colored product is obtained. This derivative is designated as α-acetate. When heated in a capillary tube it melts with decomposition at 102–104°. When the anthocyanidin chloride is refluxed with acetic anhydride for 2 hours and the reaction mixture is poured into water a second acetate is precipitated. This product is designated as β-acetate and it melts with decomposition between 220–230°.

The composition of the acetyl derivatives is shown in Table II. Both preparations were free from chlorine.



It is evident from the results given above that both acetyl derivatives contain 4 acetyl groups and slightly more than 1 methoxyl group. The products correspond therefore closely in composition to the tetraacetyl derivative of monomethoxydelphinidin but as shown below they contained a small amount of dimethoxydelphinidin, since they yielded syringic acid on oxidation.

*Oxidation of Anthocyanidin Acetates.*—When oxidized with neutral permanganate, according to the method previously described (3), the  $\beta$ -acetate yielded a very small amount of acetyl syringic acid, m.p. 187–188°, identified by mixed melting point with a sample of synthetic acetyl syringic acid. On hydrolysis of the acetate, syringic acid was obtained, m.p. 209–210°, which also gave no depression of m.p. when mixed with a sample of synthetic syringic acid.

*Analysis.* 0.1134 gm. substance: 0.2260 gm.  $\text{CO}_2$ , 0.0517 gm.  $\text{H}_2\text{O}$ .  
Calculated for  $\text{C}_8\text{H}_{10}\text{O}_6$ . C 54.54, H 5.05. Found, C 54.35, H 5.10.

The  $\alpha$ -acetate was oxidized in acetone solution with permanganate and also yielded a small amount of syringic acid identified by the mixed m.p.

By neither of the above methods was it possible to isolate a monomethoxygallic acid although the starting material contained 87 per cent as calculated from the methoxyl content of the anthocyanidin. The monomethoxygallic acid was undoubtedly destroyed by further oxidation. Further work on the decomposition of these acetates is being carried out in order to isolate the monomethoxygallic acid.

*Isolation of Delphinidin Iodide.*—The hydriodic acid solutions from the methoxyl determinations deposited on cooling dark reddish purple prisms of delphinidin iodide similar to that obtained from the other pigments studied. The crystals were filtered off, washed with ether, dried, and analyzed.

The loss in weight when dried at 105° over phosphorus pentoxide was 4.02 per cent. Calculated for a monohydrate of  $\text{C}_{15}\text{H}_{11}\text{O}_7\text{I} \cdot \text{H}_2\text{O}$ ,  $\text{H}_2\text{O}$  4.18 per cent.

*Analysis.* 0.1357 gm. substance: 0.0725 gm. Ag I.  
Calculated for  $\text{C}_{15}\text{H}_{11}\text{O}_7\text{I}$  (429.9). I 29.52 per cent. Found, 28.88 per cent.

## SUMMARY.

1. The anthocyan pigment in the American grape, Ives, has been isolated and found to consist of: (a) non-glucoside, anthocyanidin; (b) monoglucoside of dimethoxydelphinidin which is identical with oenidin; (c) monoglucoside of a monomethoxydelphinidin; (d) in addition to the above, some derivative of *p*-hydroxycinnamic acid is present.

2. The only product obtained by oxidation of the anthocyanidin acetates was syringic acid.

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## STUDIES IN THE METABOLISM OF THE BILE.

### III. THE ENTEROHEPATIC CIRCULATION OF THE BILE ACIDS.\*

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The enterohepatic circulation of the bile was first postulated by Schiff (21) in 1870. Since then the behavior of the various chief constituents of the bile has been intensely studied. Broun, McMaster, and Rous (3) and McMaster and Elman (15), who have summarized the earlier literature on this subject, affirm the enterohepatic circulation of bile pigment, but this has not been substantiated by Stadelmann (22), Hooper and Whipple (11), Whipple (25), or by Bollman, Sheard, and Mann (2). In fact the preponderance of evidence seems to be against the circulation of bilirubin. The intestinal origin and enterohepatic circulation of urobilin and urobilinogen, postulated by Müller (18) has been fully confirmed by Meyer-Betz (17), Wilbur and Addis (26), Fischler (5), Rous (19), McMaster and Elman (14), and others and is now generally accepted. That the output of cholesterol in the bile is intimately related to the intake in the food is likewise accepted (McNee (16) and McMaster (13)).

The experiments and claims of Schiff (21) in regard to the enterohepatic circulation of the bile acids have since been amply substantiated by the work of Stadelmann (22-24), Foster, Hooper, and Whipple (6), Whipple (25), Brugsch and Horsters (4), and others. Schiff gave bile orally to a dog with a biliary fistula and

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measured the changes in the total solids of the bile. Stadelmann attempted to measure the bile acids chemically but because of difficulties in the analytic method was forced to use 12 hour samples of bile. Foster, Hooper, and Whipple (6), by means of their improved method, were able to work with 2 hour samples of bile, an interval still too great for a satisfactory study of the time relations involved. Whereas the enterohepatic circulation of the bile acids is generally accepted, it should be noted that Hoppe-Seyler (12) was unable to isolate them from the blood of the portal vein.

We have been able to follow the biliary excretion of bile acids and bilirubin at half hour intervals (1, 9, 10). In consequence it has been possible to determine the curve of excretion of the bile acids after their oral administration and to compare it with the effect of intravenous injection. We have also been able, by means of our colorimetric method for the determination of the bile acids (1), to compare the changes in the blood of the portal and systemic circulations.

#### *Methods of Experimentation.*

Experiments were performed on a dog with a permanent biliary fistula prepared according to the method of Rous and McMaster (20). Samples of the hepatic bile were collected at half hour intervals. Usually two preliminary samples were collected to establish the normal rate of flow of the bile. A solution of bile salts was then given by stomach tube, in a dosage equivalent to 100 to 125 mg. of glycocholic acid for each kilo of body weight and the collection of bile at half hour intervals continued for a period of 6 hours.

Additional experiments were performed on dogs under amytal narcosis. The gallbladder was removed and a cannula placed in the common bile duct. The hepatic bile so obtained was collected to establish the normal rate of flow. Bile salts, in a dose of from 100 to 125 mg. for each kilo of body weight, were then injected into the second portion of the duodenum and the collection of bile continued at half hour intervals for a period of 6 hours.

Samples of blood were obtained from the portal vein by means of a long narrow glass cannula so inserted as not to obstruct the portal circulation. In some experiments the spleen was removed

and the cannula inserted through the splenic vein. In other experiments one of the veins from the transverse colon was used and the cannula pushed down through the superior mesenteric vein until it projected into the lumen of the portal vein. When the cannula was not in use for the withdrawal of blood, it was closed by a pipe-stem cleaner moistened with heparin solution. The injection of heparin was also found to be of advantage in preventing thrombosis within the portal vessels.

The changes observed in the blood and bile following the intravenous injection of bile salts have been reported by Greene and Snell (9). The two series of experiments have been made as nearly comparable as possible and so indicate in part the effect of the method of administration on the behavior of the bile acids within the body.

#### EXPERIMENTAL.

A series of ten animals was studied. The data obtained in two typical experiments are given in Tables I and II. The changes in these two experiments are characteristic and illustrative of the whole series. Control experiments on a fasting animal showed that following the establishment of a biliary fistula and the external drainage of the bile there is a gradual diminution in both the volume of bile and the output of bile acids.

The normal response to the oral administration of bile acids to a dog with a permanent biliary fistula is given in Table I. A slight increase in the flow of bile was observed within 30 minutes after a solution of bile salts was given through a stomach tube. The increase was progressive and the greatest outflow of bile occurred during the second and third collection periods. Thereafter the volume gradually decreased, the period of maximal choleresis lasting about 3 hours.

The effect of the oral administration of the bile acids on their excretion in the bile is best shown when the total quantity rather than the concentration is studied. There was little change during the first period of collection. Thereafter there was a striking increase which corresponded in part to the changes in the volume of the bile. The maximal excretion occurred during the second, third, fourth, and fifth periods. The excretion then fell to the level noted in the control experiments.

Similar results were obtained in the experiments carried out under amytal narcosis (Table II) although the changes were not so marked and the recovery of the injected bile acids was much less complete. This was to be expected as considerable operative manipulation was unavoidable in these experiments, and our experience has indicated that the excretion of bile by the liver was

TABLE I.

*Changes in Blood and Bile of Animal with Permanent Biliary Fistula Following the Oral Administration of Bile Acids.*

Weight of dog, 14.6 kilos.		Blood.	Bile.					Remarks.
Time of collection.			Bile acids, mg. per cent.	Volume, cc.	Bilirubin.		Bile acids.	
Before.	After.	Mg. per cent.			Total mg.	Per cent.	Total mg.	
1 hr.		7.8	5.0	19	0.95	2.63	131	Control period.
$\frac{1}{2}$ "		7.9	5.0	22	1.13	1.50	75	
	15 min.	7.4						Bile acids given, 1460 mg. Bile acids recovered, 1663 mg.
	30 "	8.3	7.5	28	2.12	1.00	75	
	45 "	8.6						
	1 hr.	8.0	15.5	16	2.52	3.36	520	
	1½ hrs.		11.0	14	1.48	4.40	485	
	2 "	7.2	6.8	15	1.00	4.00	272	
	2½ "		3.1	19	0.59	3.36	101	
	3 "	6.9	3.0	28	0.86	1.96	59	
	3½ "		3.0	37	1.10	1.26	38	
	4 "	7.7	3.3	42	1.40	0.86	38	
	4½ "		3.0	47	1.40	0.75	22	
	5 "	7.4	3.1	43	1.33	0.68	20	
	5½ "		3.1	49	1.52	0.75	23	
	6 "	7.4	2.6	56	1.45	0.75	20	

markedly affected by such manipulation, the anesthetic used, or by disturbances in the portal circulation.

Greene and Snell (9) reported that after the intravenous injection of bile salts, choleresis was most marked within 30 minutes, and the changes took place within the first three collection periods. Approximately the total quantity of injected bile acids could be

recovered in the bile within 2 hours. Comparison of our results (Fig. 1) with their Table VIII shows that while the general curve of the excretion of bile acids is similar in both series of experiments, yet when bile acids are given by mouth their appearance in the bile is delayed and the period of excretion is prolonged.

TABLE II.

*Changes in Blood and Bile Following the Oral Administration of Bile Acids.*

Weight of dog, 11.8 kilos.		Blood.		Bile.					Remarks.
Time of collection.		Bile acids, mg. per cent.		Volume, cc.	Bilirubin.		Bile acids.		
Before.	After.	Jugular.	Portal.		Mg. per cent.	Total mg.	Per cent.	Total mg.	
$\frac{1}{2}$ hr.		7.3	7.4	1.9	75	1.42	1.43	27	Control period.
	15 min.	8.0	11.8						Bile acids given, 1380 mg. Bile acids recovered, 1051 mg.
	30 "	7.9	9.6	2.5	78	1.95	2.15	54	
	45 "	8.7	8.8						
	1 hr.	8.5	9.2	2.6	76	1.98	2.94	76	
	1 $\frac{1}{2}$ hrs.	8.0	10.5	2.8	79	2.21	3.70	104	
	2 "	8.5	12.7	2.7	85	2.29	2.98	80	
	2 $\frac{1}{2}$ "			2.5	86	2.15	3.03	75	
	3 "	8.9	11.2	2.0	90	1.80	3.15	63	
	3 $\frac{1}{2}$ "			2.8	65	1.82	3.68	103	
	4 "	8.7	10.6	4.5	91	4.10	3.27	147	
	4 $\frac{1}{2}$ "			3.1	73	2.26	3.47	104	
	5 "	9.4	19.1						
	5 $\frac{1}{2}$ "			3.1	54	1.68	4.17	130	
	6 "	10.7	16.6	3.2	60	1.92	3.60	115	

This lag is evidently related to the time necessary for the absorption of the bile acids from the intestine.

The intravenous injection of a solution of bile salts produces a varying degree of hemolysis which is indicated by a subsequent increase in the excretion of bilirubin in the bile. When the bile salts are administered orally hemolysis does not occur and the excretion of bilirubin is unchanged during the experiment (Table I).

When bile acids are given orally, they are almost wholly with-

out effect on the systemic circulation. The analytic values in the peripheral blood were unchanged throughout the course of the different experiments. This was true not only when moderate doses of bile salts were given but when as much as 3 gm. for each kilo of body weight was given. When massive doses such as this are given some bile acid probably enters the peripheral circulation; various authors have reported the passage of small amounts into the urine. The quantities, however, are too small to affect the general level in the blood. In the fasting animal a significant difference did not exist between the intensity of the Pettenkofer

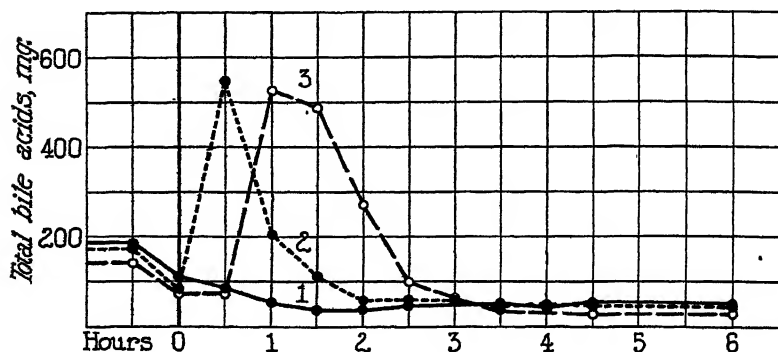


FIG. 1. Comparison of the changes in the excretion of bile acids in the bile in (1) control experiment and (2) after the intravenous administration of bile acids and (3) after the oral administration of bile acids.

reaction obtained in the arterial blood and that obtained from the jugular or portal veins.

An increase in the bile acid content of the blood from the portal vein was demonstrated within 15 minutes after the injection of a solution of bile salts into the duodenum (Fig. 2 and Table II). The analytic values were for the most part within a range of two to three times the normal. This increase in the bile acid content of the blood was not as marked as when an equivalent amount of bile acids was injected intravenously. On the other hand, the level in the blood was increased for a considerably longer time.

That this increase in the intensity of the Pettenkofer reaction obtained in the portal blood is due to the absorption of bile acids from the intestine is evidenced by Experiment 2, which shows an



accompanying increase in the flow of bile and in the output of bile acids during the period in which the level of bile acids in the blood from the portal vein was elevated. Considerable operative manipulation was unavoidable in these experiments. This not only had a definite effect on the excretion of the bile but when marked, as in the last periods of Experiment 2, permitted the passage of bile acids through the liver with a consequent terminal increase in the amount in the peripheral blood.

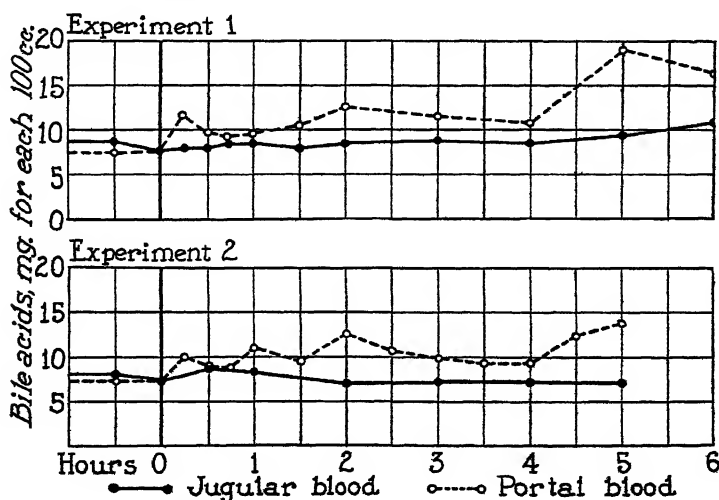


FIG. 2. The changes in the bile acid content of the blood from the jugular and portal veins following the oral administration of bile acids in Experiments 1 and 2.

We believe that under the conditions of the experiments these results can be accepted as evidence for the occurrence of bile acids in the blood from the portal vein during their absorption from the intestine. As such they form an additional link in the chain of evidence necessary to prove the presence of enterohepatic circulation of the bile acids.

#### SUMMARY.

Curves are presented to show the excretion of bile acids in the bile after their oral administration. In a dog with a biliary fistula there is a lag in the excretion as compared with the effect of intra-

venous injection that can be ascribed to absorption from the intestine.

The quantitative Pettenkofer test gives similar readings in the arterial, jugular, and portal blood of a fasting dog, but there is an increase in the amount of bile acids in the blood of the portal vein when a solution of the bile salts is injected into the duodenum. This confirms the earlier experiments of Schiff and others and extends our knowledge regarding the mechanism of the enterohepatic circulation of the bile acids.

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